USE OF ENZYME POLYMORPHISM AND HYBRIDIAZTION CROSSES TO IDENTIFY SIBLING SPECIES OF THE MOSQUITO, Anopheles quadrimaculatus (Say)

By

GREGORY CHARLES LANZARO

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THIS WORK IS DEDICATED TO THE MEMORY OF THE AUTHOR'S

FATHER, FRANK

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Abstract of Dissertation Presented to the Graduate School of the University of Florida in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

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GREGORY CHARLES LANZARO

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Work was conducted on the population genetics of the mosquito, Anopheles quadrimaculatus (Say). The research consisted of three parts: 1) electrophoretic techniques and enzyme phenotypes; 2) hybridization experiment; and 3) population genetics. Techniques were developed to visualize twenty-seven enzyme loci. The phenotypes of these are described and the inheritance patterns of nine of the polymorphic loci presented. Hybridization experiments were conducted to determine the mating compatibilities of nine geographic populations. Hybrid sterility in males produced from some of these crosses revealed the existence of two sympatic sibling species of A.quadrimaculatus at three of nine sites. Analysis of isozyme frequencies of twenty loci, also confirmed the existence of the sibling species. Genotypic frequencies of heterozygotes for alleles at two enzyme loci, Idh-1 and Idh-2, were significantly deficient for

heterozygotes at the same three localities identified in the hybridization experiment. Heterozygote deficiency was also observed at a fourth site not included in the hybridization experiment. The IDH loci were identified as being diagnostic for the two species and were used as a tool for assembling gene frequency data into discrete populations of each. An analysis of gene frequencies resulted in calculations of genetic distance between the two species, tentatively designated A. quadrimaculatus species A and B. The values obtained for genetic distance were consistent with values previously published for sibling species in the genus Anopheles.

CHAPTER I
ISOZYME PHENOTYPES AND INHERITANCE PATTERNS OF ENZYME VARIANTS IN Anopheles quadrimaculatus (Say)

Introduction

The southern house mosquito, Anopheles quadrimaculatus (Say) is one of the five species comprising the Nearctic Anopheles maculipennis complex. Work on the genetics of these species is limited. Salivary gland chromosomes have been described and polytene maps have been prepared for all the species (Kitzmiller, et al., 1967). Of the five species in the group, A. quadrimaculatus has been most studied genetically. The inheritance of DDT and dieldrin resistance have been described (Davidson, 1963; French and Kitzmiller, 1964) in this species.

In addition the inheritance of a number of morphological mutants have been described. These include stripe (French and Kitzmiller, 1963), red-stripe (Mitchell and Seawright, 1984b), black body (Seawright and Anthony, 1972) and brown body (Mitchell and Seawright, 1984a).

The karyotype of this species is comprised of two metacentric autosomes (chromosomes 2 and 3) and a pair of heteromorphic sex chromosomes (Kitzmiller and French, 1961).

Recently the mutants brown-body and stripe were assigned to chromosomes 2 and 3, respectively (Mitchell and Seawright, 1984a).

In the present study electrophoretic techniques were developed for the visualization of twenty enzyme systems representing twenty-seven enzyme gene loci. The inheritance patterns for nine of the polymorphic loci are presented. This study provides the groundwork for mapping studies using enzyme genes and a tool for the analysis of the population genetics of this species.

Materials and Methods

Gels were made using three parts Connaught starch (Connaught Laboratories Limited, Willowdale, Ontario, Canada) and one part Electrostarch (Electrostarch Company, Otto-Hiller, Madison, Wisconsin). A 12.5% (w/v) solution of the starch mixture in the appropriate gel buffer was heated over a gas flame in a 1000 ml Erlenmeyer filtration flask. The mixture was continuously swirled by hand during the entire cooking process, which generally took 4-5 minutes. When the solution came to a vigorous boil the heating process was terminated. The solution was immediately degassed by attaching the sidearm of the flask to a vacuum line. A vacuum was drawn over the solution until all small air bubbles were removed. The gel solution was then poured into a mold. The gel molds were constructed of 1/4 inch plexiglass with the top horizontal surface measuring 20 cm x

12.6 cm x 1 cm. This surface held that portion of the gel through which the samples migrated during electrophoresis. Each of the long sides of the horizontal was connected to a leg running perpendicularly, so that the entire surface formed an inverted U shape. Each of the perpendicular side walls was open along the bottom. The openings were closed with two inch masking tape when the gel was poured. A volume of 400 ml of buffer yielded a gel 1 cm thick. After pouring, the gel was cooled for about two hours at room temperature, then covered with saran wrap. The cast gels were further cooled, for at least two hours prior to loading, in a refrigerator at 5°C.

Samples were prepared for electrophoresis by first making a crude homogenate of individual adult mosquitoes. A block of 3/4 inch plexiglass containing sixty-four 1/4 inch deep wells was used to hold samples for homogenization. Each well was filled with thirty microliters of deionized water, and the block was then wrapped in saran wrap and cooled in a refrigerator for at least one hour. The block was placed in a container of crushed ice, and an individual adult mosquito was placed in each of thirty wells. The specimens were homogenized by means of stainless steel rods, which were attached to a brass plate in four rows of four rods per row. These were positioned on the brass plate so that the wells in the plexiglass block served as a template into which the sixteen rods fit. By rocking the plate rapidly from side to side sixteen samples could be homogenized simultaneously.

The homogenates were each absorbed onto 9 x 3 mm wicks cut from Whatmann 3 MM filter paper. Thirty samples and three bromphenol blue dye markers were inserted into an incision in the gel at a position 2.5 cm from the cathodal end.

Prior to loading the gels the electrode buffer chambers were filled with the appropriate electrode buffer and placed in the refrigerator. The chambers were rectangular boxes measuring 23 x 7 x 4.5 cm constructed of 1/4 inch plexiglass. The chamber was partitioned by a divider into two subchambers one 3.5 cm wide the other 2 cm wide. The smaller subchamber contained the electrode (20 gauge platinum wire) which was connected to a banana plug set in one end of the chamber. The large subchamber provided a place for the leg of the gel mold to be set. A set of two chambers, anode and cathode completed the apparatus. Each chamber held 250 ml of electrode buffer.

The loaded gel was readied for the electrophoretic run by first removing the masking tape from the openings in the legs of the gel mold and then setting each leg in an electrode chamber. This arrangement allowed current to pass through a continuous, U shaped gel so that no sponge or paper was used to connect the gel to the electrode buffer. Although this required using more starch, it provided a superior connection, since sponge or paper connectors can become dislodged or dry out.

The entire apparatus was placed in a refrigerator at 5°C to keep the gel cool. In addition, the top surface of the gel was covered with saran wrap and a plastic box containing crushed ice was placed on top, for additional cooling. Current, 125-250V, was applied to the gel by using an ISCO regulated high voltage supply unit Model 493.

Three buffer systems were required for electrophoresis of the enzymes included in this study. A description of the buffers follows:

1. CA-8 Tris-Citrate (Steiner and Joslyn, 1979)

gel buffer:	.074 M Tris (hydroxymethyl) aminomethane (Tris) .009 M citric acid pH 8.45
dilution:	none
electrode buffer:	1.37 M Tris .314 M citric acid
dilution:	Cathode; 1:3 dH_2O anode; 1:4 dH_2O

2. Ayala-C (Ayala, et al., 1972)

gel buffer:	.009 M Tris .003 M citric acid pH 7.0
electrode buffer:	.135 M Tris .040 M citric acid pH 7.0
dilution:	none

3. TC-5.5 (Selander, et al., 1971)

gel buffer:	.064 M Tris .026 M citric acid pH 5.5
dilution:	1:2 dH_2O

3. Continued.

electrode buffer:	.223 M Tris .093 M citric acid pH 5.2
dilution:	3:1 dH_2O

The buffer system used for each specific enzyme is listed in Table 1. The electrophoretic run was terminated when the bromphenol blue dye markers had migrated to the end of the gel (8.5 cm). The 1 cm thick gel was removed from the gel mold by making an incision through the leading edge, just in front of the dye marker. The gel was then cut into five, 1.5 mm thick slices by placing the gel on a plexiglass guide and using a .012 inch diameter guitar string mounted in a hack saw frame. Each slice was then stained for a particular enzyme.

Twenty enzyme systems, representing the products of twenty-seven loci were assayed. The names and Enzyme Commission numbers (E.C. No.) for each enzyme, as provided by the Commission on Biochemical Nomenclature (1972), are listed in Table 1. The abbreviation listed will be used throughout this report to indicate the enzyme system (all upper case letters) or genetic locus (only first letter capitalized).

The staining methods described below are those from Steiner and Joslyn (1979), unless otherwise noted. The quantities listed were for 50 ml of staining solution, the volume required to stain a 1.5 mm gel slice. The following

Table 1. Names, Enzyme Commission numbers, locus designations and buffer system for the enzymes assayed in this study.

Enzyme name	E.C. No.	Abbrev.	Locus	Buffer System
Acid phosphatase	3.1.3.2	ACPH	Acph	CA-5.5
Aconitase	4.2.1.3	ACON	Acon	CA-7.0
Adenylate kinase	2.7.4.3	ADK	Adk	CA-7.0
Catalase	1.11.1.6	CAT	Cat	CA-8.0
Esterase	3.1.1.1	EST	Est-1	CA-7.0
			Est-2	CA-7.0
			Est-3	CA-7.0
			Est-4	CA-7.0
Glutamate oxaloacetate transaminase	2.6.1.1	GOT	Got-1	CA-8.0
alpha-Glycerophosphate dehydrogenase	1.1.1.8	GPDH	Got-2	CA-8.0
Hexokinase	2.7.1.1	HK	Hk-1	CA-7.0
			Hk-2	CA-7.0
Hydroxyacid dehydrogenase	1.1.1.30	HAD	Had	CA-8.0
Isocitrate dehydrogenase	1.1.1.42	IDH	Idh-1	CA-8.0
			Idh-2	CA-8.0
Lactate dehydrogenase	1.1.1.27	LDH	Ldh	CA-8.0
Malic dehydrogenase	1.1.1.37	MDH	Mdh	CA-8.0
Malic enzyme	1.1.1.40	ME	Me	CA-8.0
Mannose phosphate isomerase	5.3.1.8	MPI	Mpi-1	CA-7.0
			Mpi-2	CA-7.0
Peptidase	3.4.1.1	PEP	Pep	CA-8.0
Phosphoglucomutase	2.7.5.1	PGM	Pgm	CA-8.0
6-Phosphogluconate dehydrogenase	1.1.1.43	6-PGD	6-Pgd	CA-5.5

Table 1 continued.

Enzyme name	E.C. No.	Abbrev.	Locus	Buffer System
Phosphoglucose isomerase	5.3.1.9	PGI	Pgi	CA-8.0
Sorbitol dehydrogenase	1.1.1.14	SODH	Sodh	CA-8.0
Xanthine dehydrogenase	1.2.1.37	XDH	Xdh	CA-8.0

abbreviations are used: MTT ([3-(4,5 Dimethylthiazol-2-yl) - 2,5-diphenlytetrazolium bromide]), NAD (nicotinamide adenine dinucleotide), NADP (Nicotinamide adenine dinucleotide phosphate), and PMS (phenazine methosulfate).

All reagents were purchased from Sigma Chemical Co., St. Louis, Missouri.

1. ACPH acid phosphatase: sodium alpha naphthyl acid phosphate, 50 mg; polyvinylpyrolidine, 100 mg; 0.1 M manganese chloride; 0.5 ml; sodium chloride, 500 mg; 0.05 M acetate buffer pH 5.0, 50 ml. After incubating at 37°C for 30 minutes 50 mg of Fast Blue RR was added.
2. ACON aconitase (Shaw and Prasad, 1970): cis-aconitic acid, 60 mg; 0.1 M magnesium chloride, 0.5 ml, NADP, 10 mg; isocitrate dehydrogenase, 20 units; MTT. 10 mg; 0.2 M Tris-HCl pH 8.0, 50 ml After incubation at 37°C for 30 minutes 5 mg of PMS were added.
3. ADK adenylate kinase: glucose, 200 mg; adenosine diphosphate, 40 mg; 0.1 M magnesium chloride, 5 ml; NADP, 10 mg; glucose-6-phosphate dehydrogenase, 30 units; hexokinase, 60 units ; MTT, 10 mg; PMS, 5 mg; 0.2 M Tris-HCl pH 8.0, 50 ml.
4. CAT catalase (Shaw and Prasad, 1970): 35% hydrogen peroxide, 0.1 ml; dH_2O, to 100 ml. Following incubation at room temperature for 15 minutes the

solution was drained and the gel rinsed with water. Solutions of 2% potassium ferricyanide, 25 ml and 2% ferric chloride, 25 ml were added and the mixture agitated until white bands appeared on the gel.

5. EST esterase: alpha-naphthyl acetate, 40 mg; beta-naphthyl acetate, 20 mg; 0.2 M phosphate buffer pH 6.4, 50 ml. After incubating for 30 minutes 50 mg of Fast Blue RR was added.
6. GOT glutamate oxaloacetate transaminase: L-aspartic acid, 400 mg; alpha-ketoglutaric acid, 185 mg; pyridoxal-5-phosphate, 10 mg, 0.2 M Tris-HCl pH 8.5. After incubating for 30 minutes at 37°C, 50 mg of Fast Blue RR was added.
7. a-GPDH alpha-glycerophosphate dehydrogenase: alpha-glycerophosphate, 50 mg; NAD , 20 mg; MTT, 10 mg; 0.2 M Tris-HCl pH 8.0, 50 ml. After incubating at 37°C for 30 minutes, 5 mg of PMS were added.
8. HAD hydroxyacid dehydrogenase: D-gluconic acid, 100 mg; 0.1 M magnesium chloride. 0.5 ml; sodium chloride, 100 mg; 0.2 M Tris-HCl pH 8.0, 50 ml; After incubating at 37°C for 30 minutes, 5 mg of PMS were added.
9. HK hexokinase: glucose, 50 mg; adenosine triphosphate, 40 mg; 0.1 M magnesium chloride, 1 ml; NADP, 10 mg; glucose-6-phosphate dehydrogenase, 20 units; MTT, 10 mg; 0.1 M Tris-HCl pH 7.5, 50 ml. After

incubating at 37° for 15 minutes, 5 mg of PMS were added.

10. IDH isocitrate dehydrogenase: sodium isocitrate, 50 mg; 0.1 M magnesium chloride, 2 ml; NADP, 10 mg; MTT, 10 mg; 0.1 m Tris-HCl pH 7.5, 50 ml. After incubating for 15 minutes at 37°C, 5 mg of PMS were added.

11. LDH lactate dehydrogenase: lithium lactate, 300 mg; NAD, 20 mg; MTT, 10 mg; 0.2 M Tris-HCl 50 ml. After incubating for 60 minutes at 37°C, 5 mg of PMS were added.

12. MDH malic dehydrogenase: 2.0 M DL-malate pH 7.0, 3 ml; NAD, 20 mg, MTT, 10 mg; 0.2 M Tris-HCl pH 8.0, 50 ml. After incubating at 37°C for 30 minutes, 5 mg of PMS were added.

13. ME malic enzyme: 2.0 M DL-malate pH 7.0, 2 ml; 0.1 M magnesium chloride, 2.5 ml; NADP, 10 mg; MTT, 10 mg; 0.1 M Tris-HCl pH 7.0. 50 ml. After incubating at 37°C for 30 minutes, 5 mg of PMS were added.

14. MPI mannose phosphate isomerase: (Harris and Hopkins, 1976): mannose-6-phosphate, 20 mg; 0.1 M magnesium chloride, 1 ml; NADP, 10 mg; glucose-6-phosphate dehydrogenase, 20 units; phosphoglucose isomerase, 20 units; MTT, 10 mg; 0.2 M Tris-HCl pH 8.0, 50 ml. After incubating at 37°C for 30 minutes 5 mg of PMS were added.

15. PEP peptidase: L-leucyl-tyrosine, 20 mg; peroxidase, 25 mg; amino acid oxidase, 30 mg; 0.1 M Tris-HCl pH 7.5, 50 ml. After incubating at 37°C for 30 minutes, 20 mg of 0-dianosidine-HCl was added.

16. PGM phosphoglucomutase: sodium glucose-1-phosphate, 35 mg; glucose-1, 6-diphosphate, 0.45 mg, 0.1 M magnesium chloride, 4 ml; NADP, 10 mg; glucose-6-phosphate dehydrogenase, 20 units; MTT, 10 mg; 0.1 M Tris-HCl pH 7.5, 50 ml. After incubating at 37°C for 15 minutes, 5 mg of PMS were added.

17. 6-PGD 6-phosphogluconate dehydrogenase: 6-phosphogluconate, 50 mg; 0.1 M magnesium chloride. 0.5 ml; NADP, 10 mg; MTT, 10 mg 0.1 M Tris-HCl pH 7.5, 50 ml. After incubating at 37°C for 15 minutes, 5 mg of PMS were added.

18. PGI phosphoglucose isomerase: fructose-6-phosphate, 10 mg; 0.1 M magnesium chloride, 4 ml; NADP, 10 mg; glucose-6-phosphate dehydrogenase, 10 mg; MTT, 10 mg; PMS, 5 mg, 0.1 M Tris-HCl pH 7.5, 50 ml. After incubating at 37°C for 15 minutes, 5 mg of PMS were added.

19. SODH sorbitol dehydrogenase (Shaw and Prasad, 1970): sorbitol, 250 mg; NAD 20 mg; MTT, 10 mg; 0.2 M Tris-HCl pH 8.0, 50 ml. After incubating at 37°C for 45 minutes, 5 mg of PMS were added.

20. XDH xanthine dehydrogenase: hypoxanthine, 100 mg; NAD, 20 mg; MTT, 10 mg; 0.2 M Tris-HCl pH 8.0, 50 ml. After incubating at 37°C for 30 minutes, 5 mg of PMS were added.

The name and number for enzyme loci and alleles were assigned as follows: The first letter of the locus name was capitalized. The loci were numbered in order, with the locus having the highest mobility as number one. A biochemical marker strain, called Q2, was developed by sub-culturing the ORLANDO strain of A. quadrimaculatus. With the exception of Got-2 and Mpi-2, the Q2 strain was fixed for a single allele at all the enzyme loci included in this study. Numbers were assigned to each allele based on its mobility relative to that of the allozyme found in the Q2 strain. Except for Idh-2 and Mpi-1, the Q2 allozyme (designated as 100) represented the allozyme most common in field populations. In the case of Got-2 and Mpi-2 the allele with the highest frequency in the Q2 strain was designated as 100.

Crosses to determine inheritance patterns of enzyme phenotypes were achieved using the induced copulation technique, as described by Baker et al., (1962). The Q2 strain was crossed to F_1 individuals reared from a field population located at Ginnie Springs, Florida. Both the parents and F_1 progeny were electrophoresed and stained for the various enzymes.

Results

Enzyme Phenotypes

Techniques for visualizing enzyme phenotypes needed to be developed before more detailed genetic studies could be undertaken. A variety of buffer systems were available for enzyme separation (Steiner and Joslyn, 1979; Selander, et al., 1971; Ayala et al., 1972). The systems used were obtained empirically, depending on banding quality and consistent reproducibility. The banding phenotypes are illustrated in Figures 1, 2, 3, and 4. All polymorphic loci are illustrated in these figures either by gels containing the most common allele and variants found in a field population (Figures 1 and 2) or by gels representing the results of genetic crosses (Figures 3 and 4). These observations also provided information concerning enzyme quaternary structure. Table 2 describes the number of bands observed in putative heterozygotes representing individuals from the field and/or heterozygotes resulting from genetic crosses. Enzyme structure was inferred from the number of bands present in heterozygotes (Harris and Hopkins, 1976). Nine of the enzymes(Acon, Adk, Est-1, Est-2, Est-3, Hk-1, Hk-2, Mpi-1, and Pgm) were identified as monomers which appeared as one band in homozygotes and two bands in heterozygotes. There were ten enzymes which appeared to be dimers (Got-1, Got-2, a-Gpdh, Had, Idh-1, Idh-1, Mdh, Pgi, Sodh, and Xdh). These were always presented as three-banded heterozygotes. The three banded phenotype always presented as two homodimer

Figure 1. Allozyme phenotypes of Anopheles quadrimaculatus in stained starch gel preparations. Individuals used were adults collected from a field population at Ginnie Springs, FL.

A. Aconitase. Individuals 1-3 and 7-10 are homozygous for $Acon^{100}$; 4 is an $Acon^{94/100}$ heterozygote; 5 is an Acon $^{100/106}$ heterozygote; 6 is an Acon $^{94/106}$ heterozygote.

B. Adenylate kinase. HK-1 and HK-2 commonly appeared as the series of bands positioned cathodally of the Adk bands. Individual 1 is homozygous for Adk^{117}; all others are homozygous for Adk^{100}.

C. Alpha-Glycerophosphate dehydrogenase. individual 8 is heterozygous for a-Gpdh $^{74/100}$; all others are $a\text{-}Gpdh^{100}$ homozygotes.

D. Xanthine dehydrogenase. Individuals 1, 6 and 8 are homozygous for Xdh^{105}; 4 and 5 are homozygous for Xdh^{100}; 9 is homozygous for Xdh^{108}; 2 is an $Xdh^{100/103}$ heterozygote; 3 is an $Xdh^{100/105}$ heterozygotes; 7 is an $Xdh^{103/108}$ heterozygote and 10 is an $Xdh^{100/108}$ heterozygote.

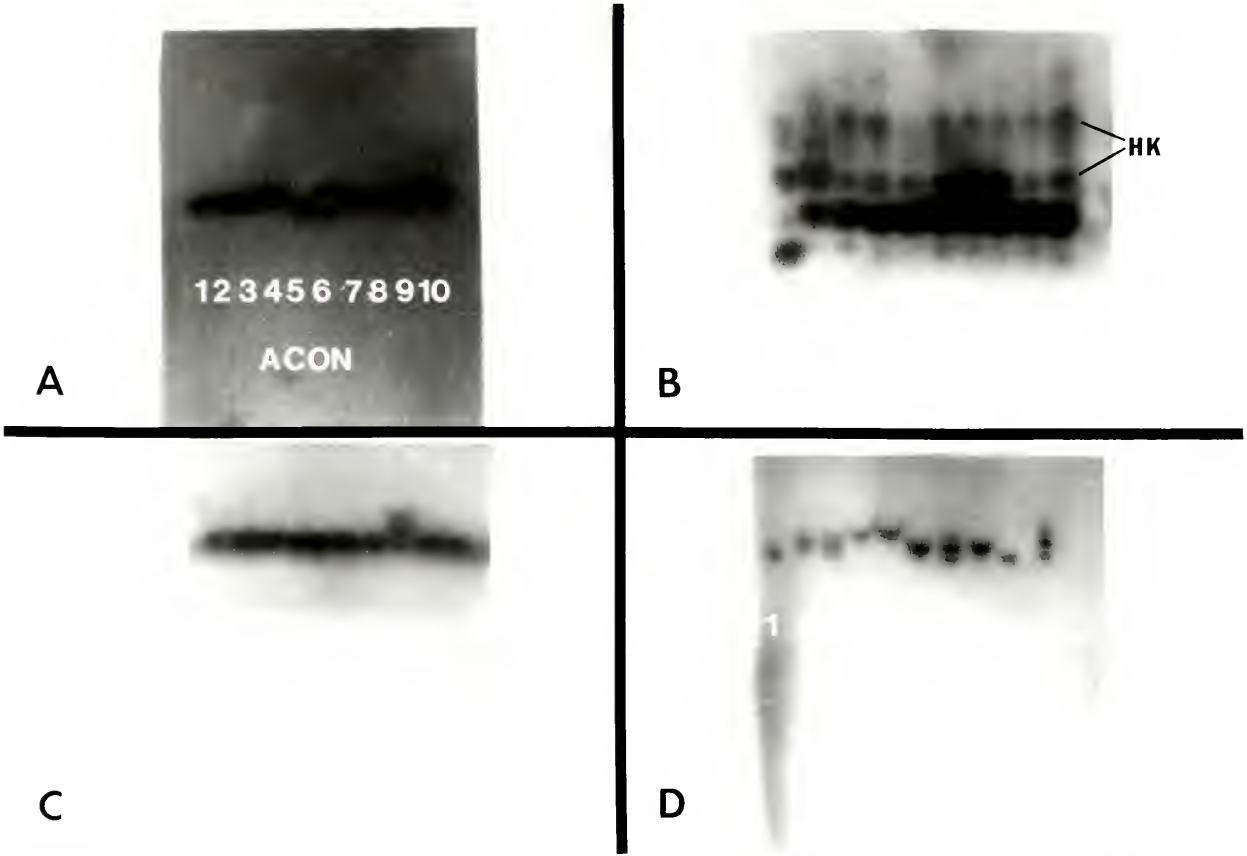

12 3 4 5 6 7 8 9 10
ACON
A
HK
B
C
1
D

Figure 2. Allozyme phenotypes of Anopheles quadrimaculatus in stained starch gel preparations. Individuals used were adults collected from a field population at Ginnie Springs, FL.
A. Glutamate oxaloacetate transaminase. Got-1: Individual 9 is a Got-$1^{100/112}$ heterozygote; all others are Got-1^{100} homozygotes. GOT-2: Individuals 2, 6, and 9 are Got-$2^{38/100}$ heterozygotes; all others are Got-2^{100} homozygotes.
B. Hydroxyacid dehydrogenase. Individuals 1-4 and 8 are Had^{100} homozygotes; 5 and 7 are Had^{156} homozygotes; 6 is a $Had^{100/156}$ heterozygote and 9 is a $Had^{56/100}$ heterozygote.
C. Hexokinase. Individual 1 is HK-$1^{100/130}$, HK-$2^{100/145}$ double heterozygote; 3 is a HK-$1^{80/100}$, HK-$2^{76/100}$ double heterozygote; all others are HK-1^{100}, HK-2^{100} homozygotes.
D. Malic enzyme. Individuals 3 and 4 and $Me^{94/100}$ heterozygotes; all others are Me^{100} homozygotes.

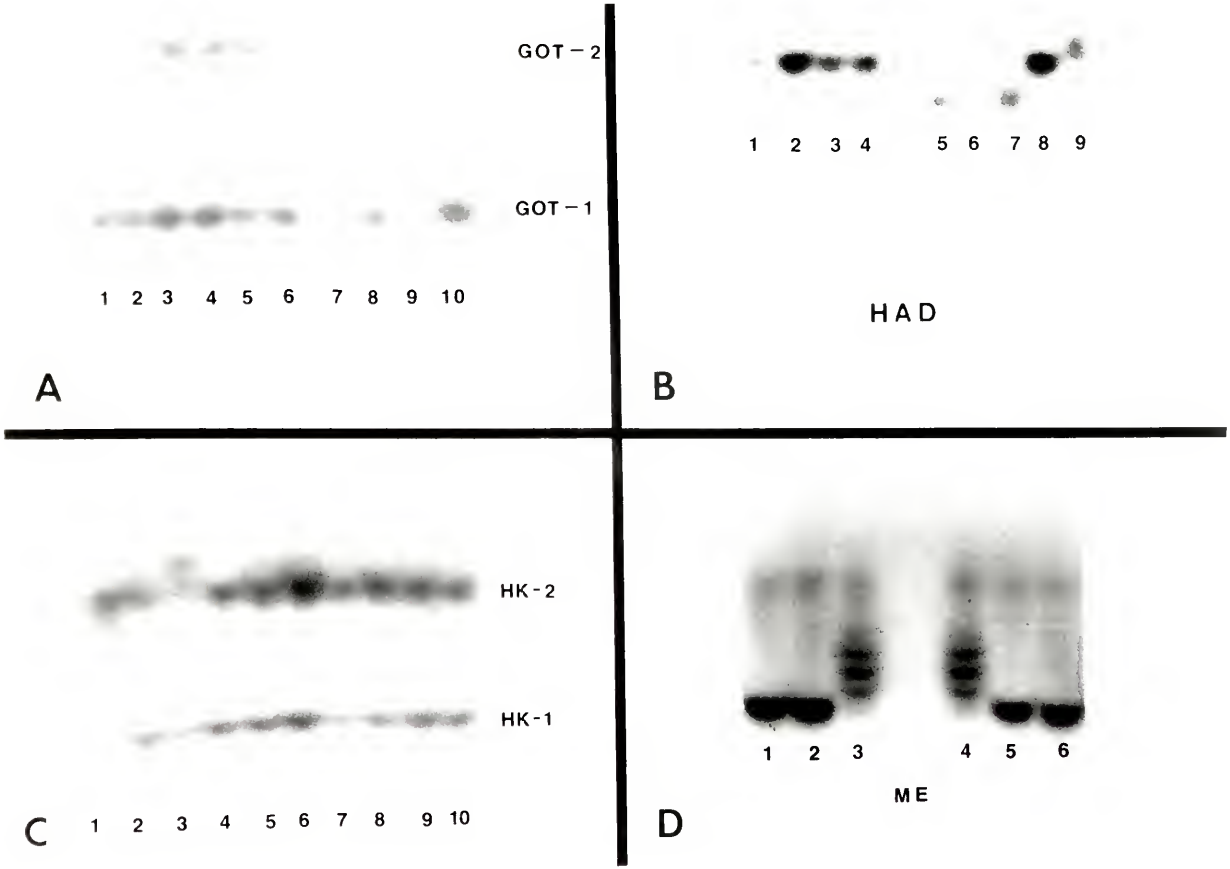

GOT – 2
GOT – 1
1 2 3 4 5 6 7 8 9 10
A
1 2 3 4 5 6 7 8 9
HAD
B
HK-2
HK-1
C 1 2 3 4 5 6 7 8 9 10
1 2 3 4 5 6
ME
D

Figure 3. Inheritance patterns of allozyme phenotypes of Anopheles quadrimaculatus. Individuals used in crosses were males from the Q2 strain force mated to females from a field population at Ginnie Springs, FL, except for MDH in which case the reciprocal cross was used. Individuals 1 and 2 are parental female and male, respectively; individuals 3-7 are F_1 females; 8-12 are F_1 males.
A. Esterase. Est-1: all individuals are homozygous for Est-1^{100}; Est-2; Individual 1 is an Est-$2^{100/105}$ heterozygote, 2 is homozygous for Est-2^{100}, 3-5, 8, 11 and 12 are Est-2^{100} homozygotes; 6, 7, 9 and 10 are Est-$2^{100/105}$ heterozygotes. Est-3; all individuals are Est-3^{100} homozygotes. Est-4: Individual 1 is an Est-4^{103} homozygote, 2 is an Est-4^{100} homozygote, 3-12 are Est-$4^{100/103}$ heterozygotes.
B. Isocitrate dehydrogenase. Idh-1: Individual 1 is an Idh-1^{85} homozygote, 2 is an Idh-1^{100} homozygote, 3-12 are Idh-$1^{100/85}$ heterozygotes. Idh-2: Individual 1 is an Idh-$2^{136/173}$ heterozygote, 2 is an $Idh^{10}0$ homozygote, 3-6 and 9-11 are $Idh^{100/136}$ heterozygotes, 7, 8, and 12 are $Idh^{100/173}$ heterozygotes.
C. Malic dehydrogenase. Individual 1 is an Mdh^{100} homozygote, 2 is an $Mdh^{100/125}$ heterozygote, 3, 6, 7, 10 and 11 are Mdh^{100} homozygotes, 4, 5, 8, 9 and 12 are $Mdh^{100/125}$ heterozygotes.
D. Phosphoglucose isomerase/Phosphoglucomutase. PGI: All individuals are homozygous for the Pgi^{100} allele. PGM: Individual 1 is a $Pgm^{100/86}$ heterozygote, 2 is a Pgm^{100} homozygote, 5,9,10 and 11 are Pgm^{100} homozygotes, 3, 4, 6, 7, 8 and 12 are $Pgm^{100/86}$ heterozygotes.

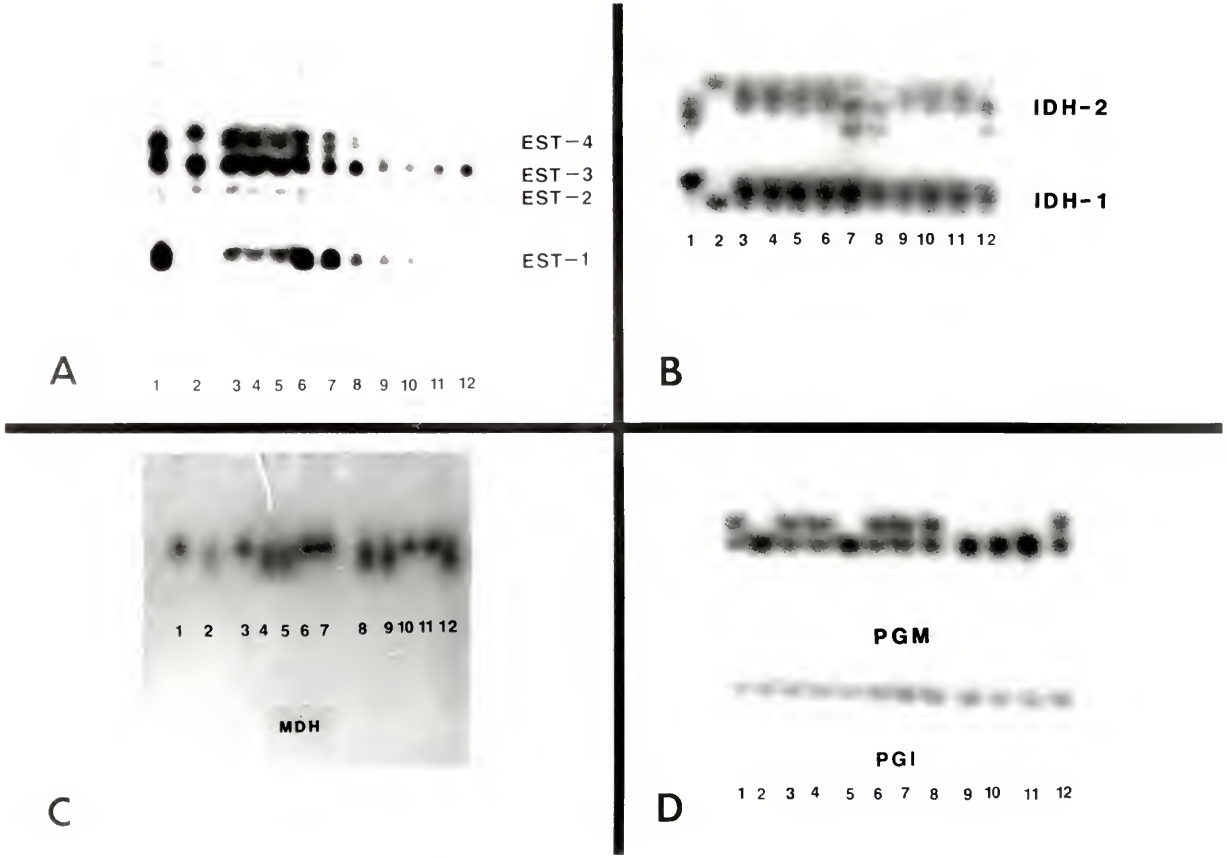

EST-4
EST-3
EST-2
EST-1
1 2 3 4 5 6 7 8 9 10 11 12
A
IDH-2
IDH-1
1 2 3 4 5 6 7 8 9 10 11 12
B
1 2 3 4 5 6 7 8 9 10 11 12
MDH
C
PGM
PGI
1 2 3 4 5 6 7 8 9 10 11 12
D

Figure 4. Inheritance patterns of allozyme phenotypes of Anopheles quadrimaculatus.

A. Glutamate oxaloacetate transaminase. Individual 1 is the parental female, 2 is the parental male, 3-7 are F_1 females, 8-12 F_1 males. Individual 1 is a Got-1^{100} homozygote, 2 is a Got-$1^{100/84}$ heterozygote, 4, 7, 8, 9 and 11 are Got-1^{100} homozygotes, 3,5,6,9 and 12 are Got-$1^{100/84}$ heterozygotes.

B. Malic enzyme. Inheritance pattern shows the Me locus is sex-linked. Individuals 1 and 2 are respectively the parental female and male, 13 and 14 are likewise parental female and male for the reciprocal cross. Individuals 3-7 and 15-19 are F_1 females, 8-12 and 20-24 are F_1 males. Individual 1 is homozygous for the Me^{100} allele, 2 is hemizygous for the Me_94 allele, 3-7 are $Me^{100/94}$ heterozygotes, 8-12 are hemizygous for the Me^{100} allele. Individual 13 is an Me^{94} homozygote, 14 is hemizygous for the Me^{100} allele, 15-19 are $Me^{100/94}$ heterozygotes, 20-24 are hemizygous for the Me^{94} allele.

C. Mannose phosphate isomerase. Inheritance pattern shows the Mpi-1 locus is sex-linked. Individuals 1 and 2 are respectively parental female and male, 3, 4 and 7, 8 are F_1 females. Individual 1 is homozygous for the Mpi-1^{92} allele, 2 is hemizygous for the Mpi-1^{87} allele, 3, 4, 7 and 8 are Mpi-$1^{92/87}$ heterozygotes, 5, 6, 9 and 10 are hemizygous for the Mpi-^{92} allele.

D. Peptidase. Individuals 1 and 2 are respectively parental female and male, 4 and 5 are likewise parental female and male. Individual 3 is an F_1 female from the cross between 1 and 2, 6 is an F_1 male from the cross between 4 and 5. Individuals 1 and 5 Pep^{50} homozygotes, 2 and 4 are Pep^{100} homozygotes, 3 and 6 are $Pep^{100/50}$ heterozygotes.

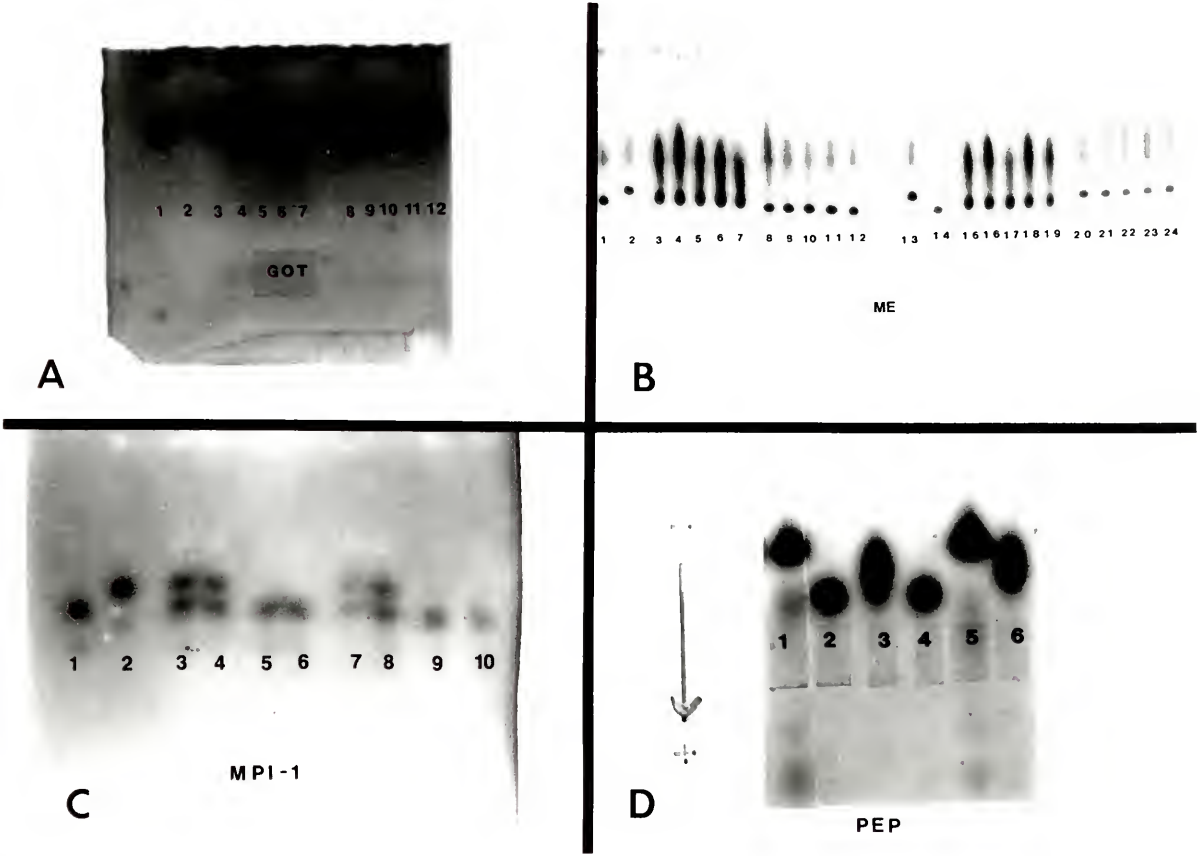

1 2 3 4 5 6 7 8 9 10 11 12
GOT
A
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
ME
B
1 2 3 4 5 6 7 8 9 10
MPI-1
C
1 2 3 4 5 6
PEP
D

Table 2. Description of enzyme phenotypes and evidence for polymeric structure of 11 of 20 loci in Anopheles quadrimaculatus. NI = not investigated.

Locus	No. of electromorphs in gel phenotype: Maximum per individual	Present in heterozygotes of genetic cross	Inferred structure
Acon	2	NI	monomer
Adk	2	NI	monomer
Est-1	2	2	monomer
Est-2	2	2	monomer
Est-3	2	2	monomer
Got-1	3	3	dimer
Got-2	3	3	dimer
a-Gpdh	3	NI	dimer
HK-1	2	NI	monomer
HK-2	2	NI	monomer
Had	3	NI	dimer
Idh-1	3	3	dimer
Idh-2	3	3	dimer
Mdh	3	3	dimer
Me	5	5	tetramer
Mpi-1	2	2	monomer
Pgm	2	2	monomer
Pgi	3	NI	dimer
Sodh	3	NI	dimer
Xdh	3	NI	dimer

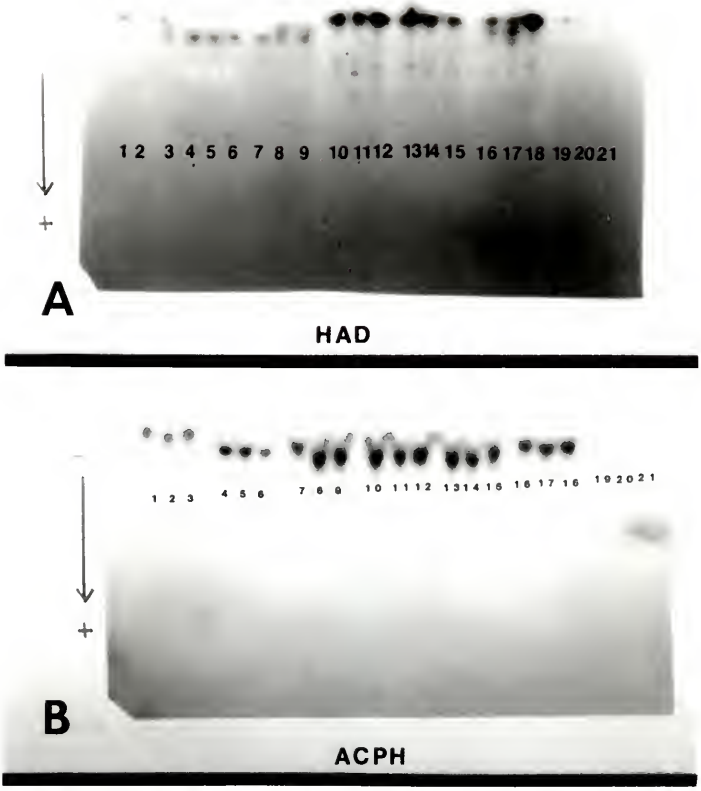
bands migrating to the same position on the gel as the respective homozygotes and a denser, hybrid band at a position between the two homodimers (see especially figure 3B). One enzyme Me presented a five banded heterozygote (Figure 2D) phenotype indicating a tetrameric structure. Observations concerning enzyme structure are only inferences. Definitive determinations require enzyme purification and dissociation-reassociation studies.

Unusual and Epigenetic Effects

Some banding patterns were observed which either did not appear consistently or which failed to give predicted results when studied by genetic crossing. In every gel stained for Adk two rows of bands appeared cathodally to the major Adk bands (Figure 1B). By comparing these to gels containing the same material and stained for HK, it was determined that these bands represented the two HK loci (Figure 2C). In addition, a band located anodally to the Adk bands was sometimes present (Figure 1B).

The most interesting effects were observed in three enzymes, Had, Acph, and 6-Pgd and were related to blood feeding in females. Figure 5 illustrates the effect of blood feeding on the electrophoretic mobility of these three enzymes. In Had the mobility and intensity of the band were increased in females 24 hours after blood feeding (Figure 5A). Mobility returned to normal by 72 hours after blood

Figure 5. The effect of blood feeding on the phenotype of certain enzymes in Anopheles quadrimaculatus. All individuals used were from the Q2 strain and were genotypically identical for the three enzyme loci illustrated. The individuals in the gels shown were treated as follows: individuals 1-3 = non-bloodfed females; 4-6 =females 24 hours after taking a blood meal; 7-9 = 48 hours post blood meal; 10-12 = 72 hours post blood meal; 13-15 = 96 hours post blood meal; 16-18 following oviposition; 19-21 = males.
A. Hydroxyacid dehydrogenase. Individuals 1-3 normal position of the Had^{100} allele in non-blood fed females, 4-9 increased mobility and intensity of staining in females analyzed 24-48 hours following a blood meal; 10-18 return to normal mobility, but with increased intensity by 72 hours following a blood-meal, 16-18 normal mobility with some smeariness in females following oviposition, 19-21 normal mobility and faint bonding in adult males.
B. Acid phosphatase. Individuals 1-3 normal mobility for the $Acph^{100}$ allele in non-blood fed females, 4-6 increased mobility and staining intensity in females 24 hours after taking a bloodmeal, 7-15 mobility increased further and bands smeary in females 48-96 hours post-bloodmeal, 16-18 mobility decreased, but not at normal position and bands compact in females following oviposition, 19-21 normal mobility and weak banding in adult males.
C. 6-Phosphogluconate dehydrogenase. Individuals 1-3 normal mobility of the $6\text{-}Pgd^{100}$ allele in non-blood fed females, 4-6 increased mobility and staining intensity in females 24 hours after taking bloodmeal, 7-15 increased mobility, stain intensity and smeariness in females 48-96 hours post-bloodmeal, 16-18 return to normal mobility with increased staining intensity in females following oviposition, 19-21 normal mobility and weak staining in adult males.


1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21
+
A
HAD
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 16 19 20 21
+
B
ACPH
1 2 3 4 5 6 7 8 9 16 17 18 19 20 21
+
C
6 – PGD

feeding, although staining intensity was higher than in non-bloodfed females. A similar effect was observed in 6-Pgd (Figure 5C). In this case mobility and intensity increased 24 hours after blood feeding, by 48 hours mobility remained higher, but the banding became more diffuse. The diffuse banding persisted through 96 hours after blood feeding. Following oviposition the banding pattern returned to normal, but staining was still more intense. The most profound effect was observed in Acph (Figure 5B). The pattern was similar to that observed in 6-Pgd. Increased mobility at 24 hours after a bloodmeal with diffuse banding at 48-96 hours post bloodmeal. However in this case the smeariness disappeared after oviposition, but the mobility remained higher than in females which never had a bloodmeal.

Discussion

Electrophoretic techniques for the analysis of twenty-seven enzyme gene loci were developed. Results revealed genetic variability at twenty of the twenty-seven loci. Inheritance patterns were determined for nine of the polymorphic loci.

Epigenetic effects on three loci (Acph, Had and 6-Pgd) were shown to be related to blood feeding in females. These effects alter the mobility of these isozymes and should be considered when interpreting electromorphs.

These techniques can now be applied to studies on genetic mapping and population genetics of A. quadrimaculatus.

CHAPTER II

EXPERIMENTAL HYBRIDIZATION OF GEOGRAPHIC STRAINS OF *Anopheles quadrimaculatus* (Say)

Introduction

Species in the genus *Anopheles* commonly evolve without developing conspicuous morphological differences. An increasing number of sibling species are being described in this genus from throughout the world, as documented by numerous authors (see Discussion section, this paper).

Hybridization studies have been widely used to establish the true biological species status of suspected sibling species (Davidson, 1964, Davidson and Hunt, 1973, Paterson et al., 1963). In addition, hybridization experiments have been used to assess the degree of relatedness between sibling and morphologically distinct, but related species (Davidson et al., 1967, Kitzmiller et al., 1967).

The first sibling species complex described in the genus *Anopheles*, was the *Anopheles maculipennis* complex. The complex contains both Palearctic (Old World) and Nearctic (New World) species. The Palearctic members include the nine

sibling species, Anopheles atroparvus Van Thiel, A. beklemishevi Stegnii and Kabanova, A. labranchiae Falleroni, A. maculipennis Meigen, A. martinius Shingarev, A. melanoon Hackett, A. messae Falleroni, A. sacharovi Farre and A. sicaulti Roubaud (White, 1978). The Nearctic members of the complex are morphologically distinct and include A. aztecus Hoffmann, A. earlei Vargus, A. freeborni Aitken, A. occidentalis Dyar and Knab and A. quadrimaculatus Say (Buonomini and Mariani 1953, Kitzmiller et al., 1967). Kitzmiller (1977) used polytene banding patterns to place A. quadrimaculatus in a separate group that included Anopheles walkeri Theobald and Anopheles artopos Dyar and Knab. However, Joslyn (1978) recorded only nonviable eggs from crosses with those species. On the contrary, viable eggs were obtained from crosses of A. quadrimaculatus to A. freeborni and A. aztecus (Kitzmiller, et al., 1967). These results indicate a closer relationship between A. quadrimaculatus and members of the Maculipennis complex, instead of A. atropos or A. walkeri.

To date no sibling species have been described in the Nearctic Anopheline fauna. A number of the Nearctic species have broad distributions, and given what is known about other species in this genus, make ideal subjects for studies on the genetics of speciation. The purpose of the present study was to assess the degree of cross fertility among nine field populations of A. quadrimaculatus. This species has a broad

geographic distribution, ranging over the entire eastern half of the United States.

Materials and Methods

The mosquitoes used in matings were offspring of females collected from the following sites: in Florida at Ginnie Springs (GIN), Gainesville at Kanapaha Botanical Gardens (KBG), and Lake Panasofkee (PAN); in Alabama, 41 miles west of Auburn on I-85 (AUB), and Guntersville (GUN); in Mississippi at Skene (SKE); in Arkansas at Stuttgart (ARK) and Bebee (BEB); and in Louisiana at Lake Charles (LAC) (Figure 6).

Field Collections

Both sexes were collected from daytime resting sites, e.g. treeholes, farm buildings, and boxes placed in wooded areas. Adults were put in Savage cages (Savage and Lowe, 1971) and provided with a 10% sucrose solution. The cages were placed in styrofoam ice chests containing a small amount of ice in plastic bags to keep the mosquitoes cool and humidified. The chests were then air-mailed or transported by car back to the laboratory.

Laboratory Procedures

On arrival, adults were transferred to larger cages (1 meter square). Gravid females from each collection were transferred individually to 30 dram containers for oviposition and non-blooded females were provided with bloodmeals by placing a confined guinea pig in the cage overnight. Blooded females were removed, held for ovarian

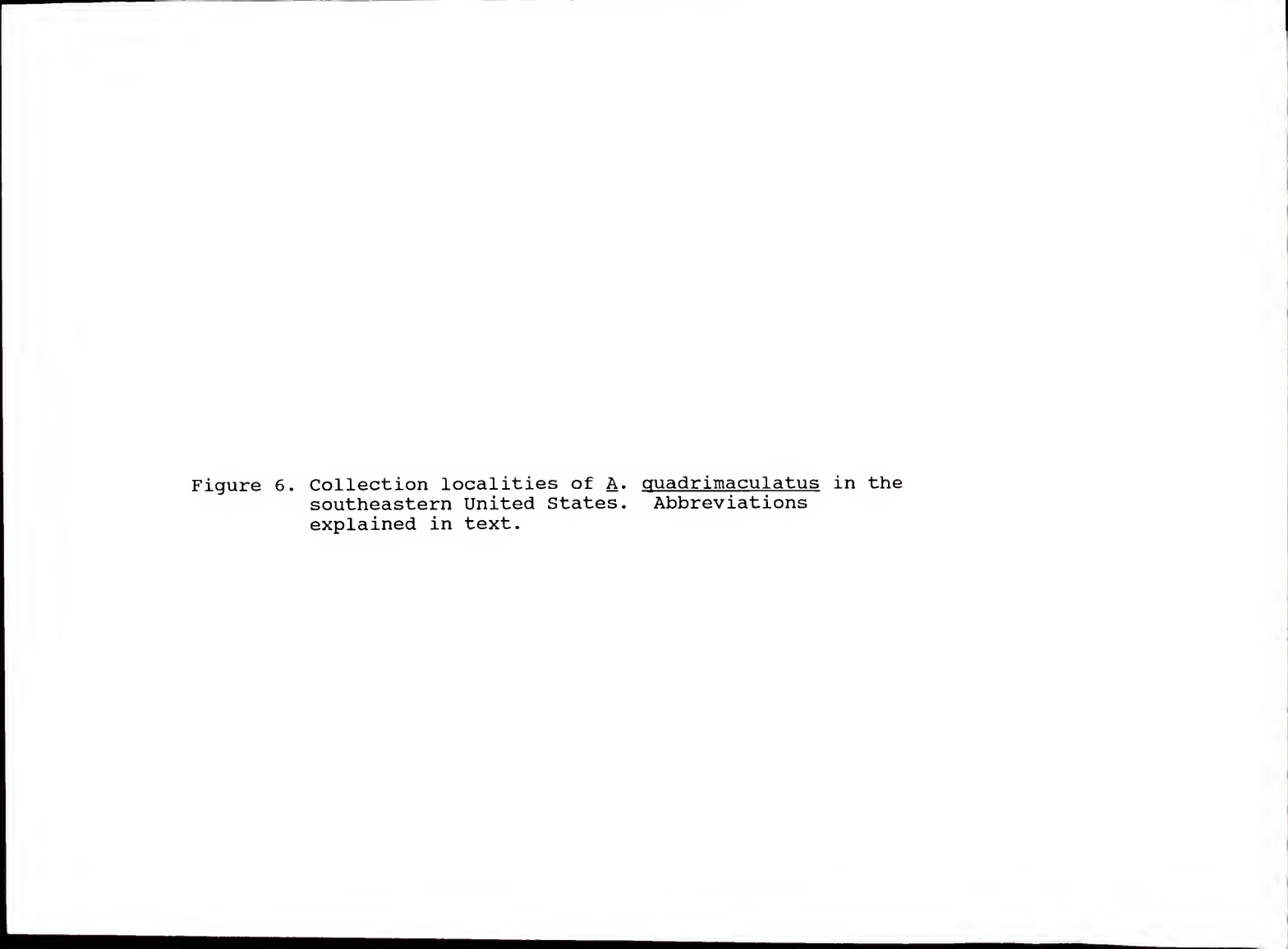
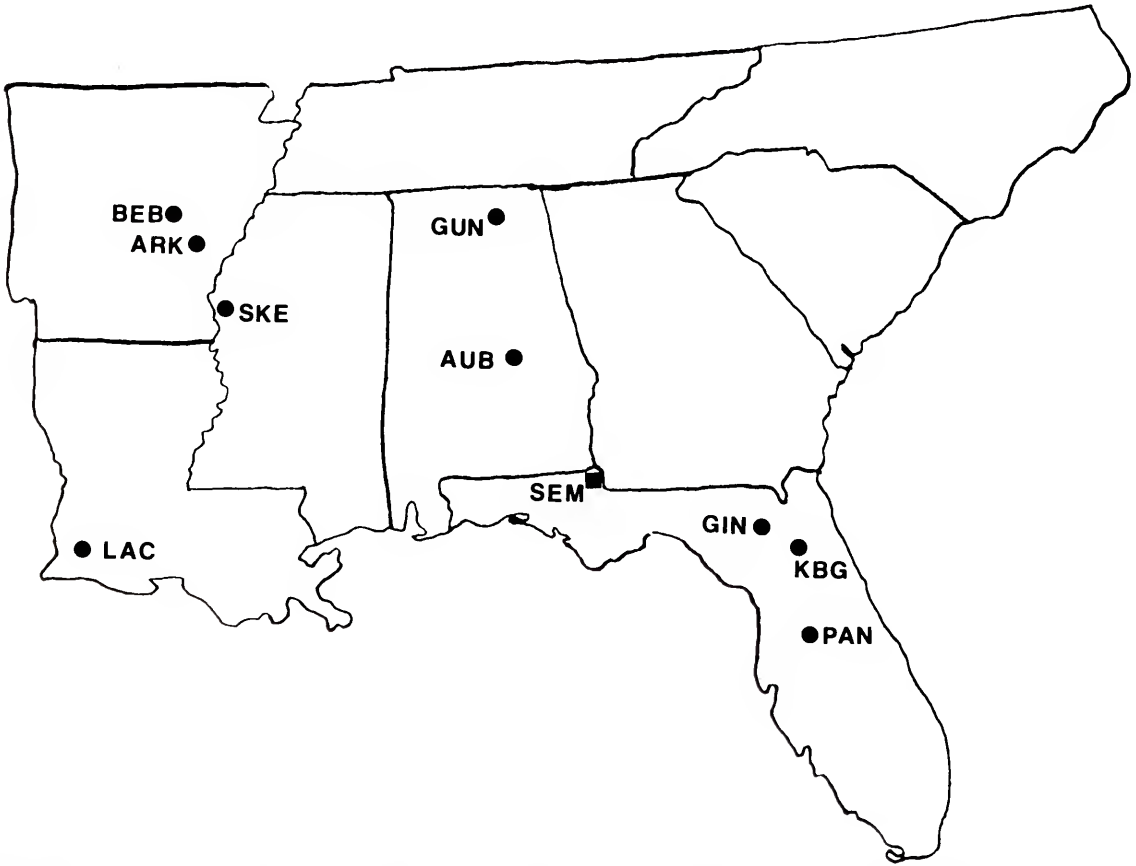


Figure 6. Collection localities of A. quadrimaculatus in the southeastern United States. Abbreviations explained in text.


BEB
ARK
SKE
GUN
AUB
SEM
LAC
GIN
KBG
PAN

development and placed in containers for oviposition. Frequently, females would not oviposit, even though they were obviously gravid. Females were traumatized to induce oviposition by tearing one wing from the thorax with a jewelers forceps. Following oviposition, females were removed and the eggs left in the containers to hatch, usually 1 to 2 days after they were laid. Newly hatched eggs were infused with 1/2 ml. of a 2% aqueous suspension of 2 parts Tetramin Baby "E" fish food and 1 part brewer's yeast.

One day after hatching the larvae were transferred to large (40.6cm X 50.8cm X 7.6cm) plastic trays in 2 to 2.5 liters of tap water. Larvae from females collected from the same site were pooled. Each tray contained about 300 larvae and the larvae were fed daily on 20 ml of the mixture described above. Larvae were reared at 27°C and pupation usually began within a week of hatching. Pupae were removed from trays and placed in 8 oz. plastic cups half-filled with tap water. The cups were capped with 1 pint cardboard ice cream containers covered with a mesh lid that provided a place for emerging adults to collect. Adults emerged about 36 hrs post-pupation. Newly emerged adults were removed, sexed, and placed in Savage cages. The adults were maintained at 25°C and 70-80% RH and were provided with a 10% sucrose solution. The numbers of F_1 adults obtained from field collected females were adequate for the completion of all the crosses, so that further maintenance of stocks by

inbreeding was not necessary. The initial series of crosses were all between the F_1 adults and ORLANDO (ORL), a standard laboratory strain maintained over forty years. The ORL strain served as the standard against which all field strains were compared. All crosses were accomplished using a modification of the induced mating technique of Baker et al. (1962). Females were held for 2 to 5 days prior to mating. In order to avoid wasting time mating females which might subsequently refuse to take a bloodmeal, females were bloodfed on guinea pigs or humans immediately prior to mating. Sterility in hybrid males was determined by microscopic examination of the testes. The testes and the distal portion of the vasa deferentia were dissected out and transferred to a small drop of saline on a slide. A cover slip was added and gentle pressure was applied. The preparation was examined at 400x. Sterility could be detected by the absence of normal spermatozoa, and could usually be predicted by the gross appearance of the testes which were greatly reduced in size in most sterile individuals. Sterility in females was tested by crossing to fertile males.

Development of strains of sibling species A and B.

Four strains from the AUB and KBG populations were developed for further study. These strains were selected on the basis of the fertility of the F_1 progeny obtained in crosses to the ORL strain (i.e., produced fertile or sterile

hybrid males). A series of isofemale lines were established from field collected females from the AUB and KBG sites. A sample of F_1 males from each line was mated to ORL females and the remainder of the F_1's were sib-mated. The adult males produced from the crosses to ORL were scored for fertility. Lines which produced fertile hybrid males were pooled and maintained as the A-strains and those which produced sterile hybrid males were combined to make the B strains. Thus two pairs of sympatric lines, AUB-A and AUB-B and KBG-A and KBG-B, were developed.

Results

Survey of Field Populations

The results from the first series of crosses, involving matings between field strains and ORL mosquitoes, are presented in Tables 3 and 4. These data represent only egg batches which hatched. A significant number of females from all crosses laid egg batches which failed to hatch. This phenomenon is undoubtedly due, in part, to the use of the induced mating technique since induced matings often result in copulation without the transfer of sperm (Bryan, 1973). These infertile matings appear normal, but the females are not inseminated and lay only infertile eggs.

Results revealed the presence of two types of individuals from the field, designated type A and type B. Type A individuals were genetically compatible with ORL, type B individuals were incompatible with ORL. The populations

Table 3. The percentage hatch, sex ratio, percent survival to adult stage and F_1 male fertility in Type A population cross-matings.

Cross female and male	Number of egg batches	Percent hatch	Total adults	Percent males	Percent survival to adult stage	Male fertility
CONTROL						
ORL x ORL	5	85.4	796	49.4	77.9	+
BEB x ORL	9	88.7	1332	52.5	78.3	+
ORL x BEB	9	85.9	870	51.5	90.0	+
ORL x GIN	8	91.7	883	56.3	91.4	+
GUN x ORL	11	86.0	1698	52.3	86.7	+
ORL x GUN	10	82.8	1088	48.2	82.9	+
LAC x ORL	10	91.7	2320	51.8	92.3	+
ORL x LAC	16	85.3	1359	52.4	88.1	+
ORL x PAN	8	78.7	700	55.7	77.4	+
SKE x ORL	7	87.7	968	51.7	86.1	+
ORL x SKE	8	80.8	1118	48.2	82.4	+

Table 4. The percentage hatch, sex ratio, percent survival to adult stage and F_1 male fertility in Type A/B population cross-matings.

Cross female X male	Number of egg batches	Percent hatch	Total adults	Percent males	Percent survival to adult stage	Male fertility
A-ARK x ORL	12	71.4	1132	51.9	81.3	+
ORL x A-ARK	13	80.9	1530	51.0	75.3	+
B-ARK x ORL	2	66.4	125	54.4	78.1	-
ORL x B-ARK	12	74.5	799	27.8	55.5	-
ORL x A-AUB	20	nd	2723	55.0	nd	+
B-AUB x ORL	10	70.1	982	51.4	70.3	-
ORL x B-AUB	12	71.3	526	12.2	34.9	-
A-KBG x ORL	6	83.3	519	48.8	66.1	+
ORL x A-KBG	7	85.8	643	50.2	63.0	+
ORL x B-KBG	7	nd	447	21.3	nd	-

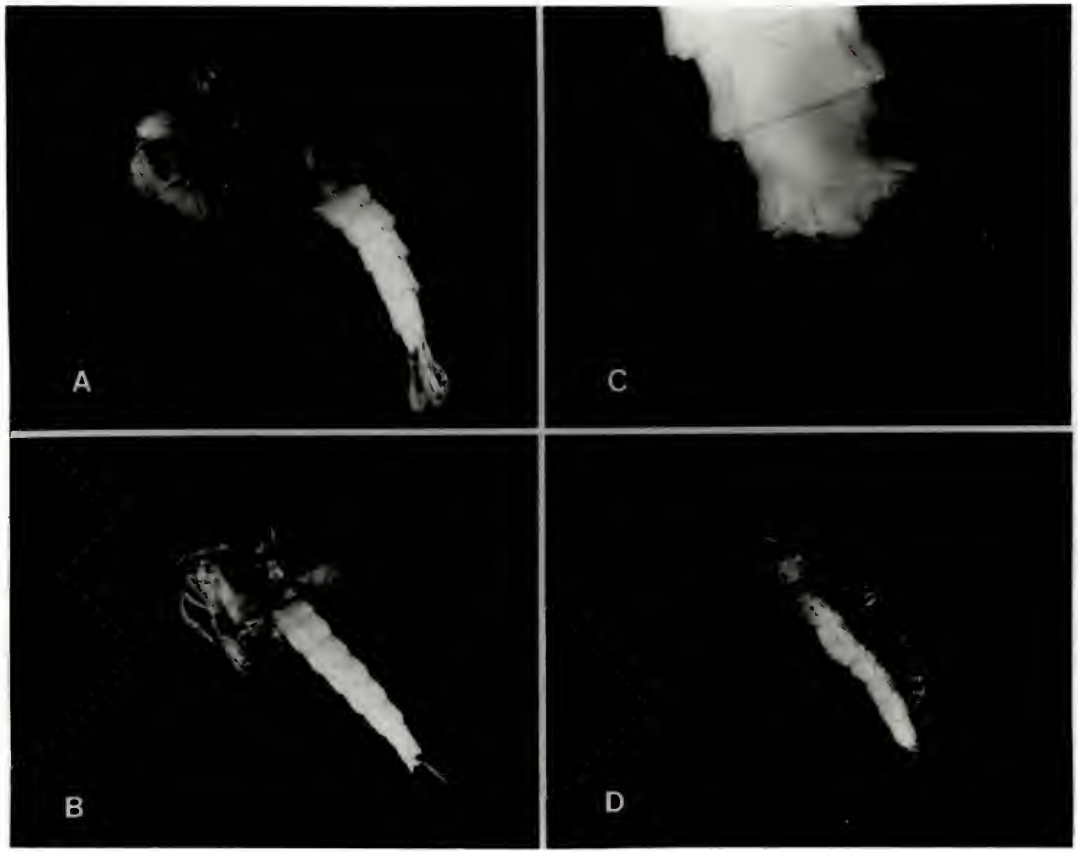
nd: Data not determined.

were divided into two groups: type A populations, comprised entirely of type A individuals and type A/B populations, which were made up of a mixture of type A and type B individuals. Crosses were made between all of the field populations and ORL but all possible reciprocal crosses were not achieved.

BEB, GIN, GUN, LAC, PAN and SKE were type A populations. When crossed to ORL the F_1 progeny were normal in every respect and the results were consistent with the control ORL x ORL crosses (Table 3). Crosses between individuals from these six populations and ORL produced families with high hatch (78.7 - 91.7%), high survival to adult stage (77.4 - 92.3%), 1:1 sex ratio (% males = 48.2 - 56.3) and fertile male progeny. The percent survival to adult stage was generally higher in the hybrid F_1 than in the control; the average for the ten hybrid crosses was 85.6% compared with 77.9% in the control. In outcrossing a longstanding colony strain to field material one might expect increased vigor in the F_1 resulting from heterosis. The populations ARK, AUB and KBG are type A/B populations. Two types of results were obtained from matings to ORL. Some crosses were identical in outcome to those from the A populations, while others resulted in the production of sterile males in the F_1. These data were grouped into A and B crosses, and are presented as such in Table 4. Crosses in which B females were mated to ORL males were completed for the ARK and AUB populations. In these crosses, hatch was high, survival to adult stage was

high and sex ratio was normal. In both crosses all F_1 males were sterile. The reciprocal cross, ORL female X B male was done for the ARK, AUB and KBG populations. Hatch was high in the ARK and AUB crosses, but was not recorded for the KBG crosses. Percent survival to the adult stage was significantly lower in the ORL X B-ARK and ORL X B-AUB crosses than in the respective reciprocal crosses. This was due to heavy mortality in the male pupae. Consequently, the sex ratio in the F_1 was strongly distorted in favor of females, 27.8% males in the ORL X B-ARK cross, 12.2% males in the ORL X B-AUB cross and 21.3% males in the ORL X B-KBG cross. The abnormal male pupae produced from these crosses are illustrated in Figure 7. Typically, in these pupae the wing buds lie outside the cephalothoracic capsule (Figure 7A). The wing buds became swollen with water and presumably disrupted the pupa's ability to maintain buoyancy. In some pupae, the head and thoracic appendages as well as the wing buds were free (Figure 7B). In addition to these abnormalities some of the male pupae had deformed genitalia. Normally, the pupal genitalia lie in a genital pouch, and in males this pouch is somewhat pointed and bifurcated distally, with lobes being equal in size. In some of the hybrid male pupae one or both lobes were not developed (Figure 7C). Many of the pupae which did survive through the pupal stage died during eclosion. Figure 7D shows a typical case where the pupa has freed its abdomen from the puparium, but was unable to free its head and thorax. No abnormalities were observed

Figure 7. Abnormal hybrid male pupae from the cross ORL female x Type B male.
A. Hybrid pupa with wing buds free of cephalothoracic capsule.
B. Hybrid pupa with head and thoracic appendages free of cephalothoracic capsule.
C. Abnormal genitalia in hybrid pupa: Asymmetry in the lobes of the genital pouch.
D. Partially eclosed male pupa. Adult abdomen is free of puparium, but head and thorax remain trapped.


A
C
B
D

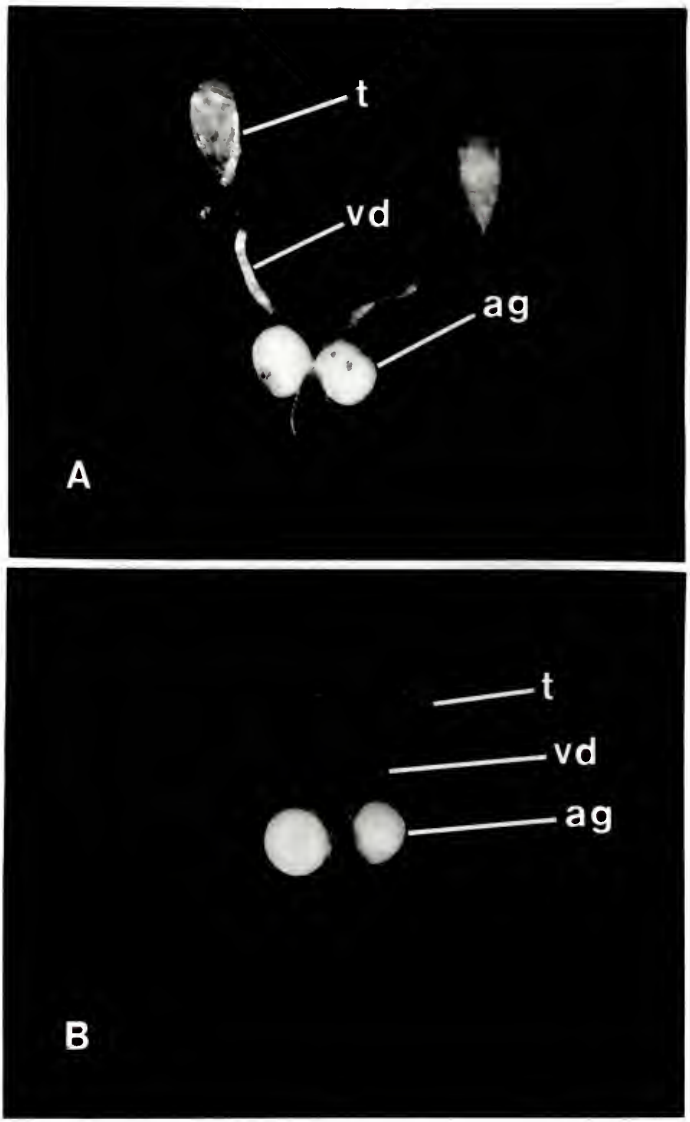
among the female pupae. Without exception all the hybrid males in each family from crosses between ORL and B males were sterile. Figure 8 compares the gross appearance of the normal and hybrid male reproductive systems. In normal males, the testes were ovoid and larger than the accessory glands (Figure 8A). The appearance of the hybrid male reproductive system varied considerably. In many cases the testes were completely atrophied and no wider than the vasa deferentia. On the other extreme, some hybrid males had testes which were normal in size, but contained no normal spermatozoa. Figure 8B shows the reproductive system of a hybrid male, the testes are smaller than the accessory glands, and contained no sperm. Figure 9 shows the contents of normal (9A) and hybrid (9B) testes. A large ball of spermatozoa has been extruded through the vas deferens of the normal testis (Figure 9A). A few abnormal sperm with short tails can be seen in the hybrid testis (Figure 9B).

Hybridization of A and B Strains

A second series of crosses were undertaken using A and B strains developed from both the AUB and KBG populations. Crosses were done to define, more completely, the relationship between type A and type B individuals. The results from these crosses are presented in Tables 5 and 6.

Data from matings within each of the four strains were collected to establish the integrity of each strain and to provide data to which hybrid crosses could be compared. As

Figure 8. The male reproductive system of A. quadrimaculatus
A. Normal male reproductive system
B. The male reproductive system, showing reduced testes from a hybrid produced by crossing ORL female and type B male.
t = testis; a.g. = accessory gland; v.d. = vas deferens


t
vd
ag
A
t
vd
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B

Figure 9. Testes of A. quadrimaculatus
A. Normal testis showing a full complement of mature spermatozoa.
B. Testis from hybrid male produced from the cross, ORL female x type B male. This testis contained a few abnormal sperm but was devoid of normal spermatozoa.

B

Table 5. The percentage hatch from A and B strains and cross-matings.

Cross female and male	Number of egg batches	Total eggs	Percent hatch
AUB-A x AUB-A	5	1376	79.8
AUB-B x AUB-B	12	2030	73.9
AUB-A x ORL	7	1022	83.1
ORL x AUB-A	7	1136	89.1
AUB-B x ORL	13	2119	78.4
ORL x AUB-B	6	756	92.7
AUB-A x AUB-B	12	3087	78.6
AUB-B x AUB-A	15	3495	90.9
KBG-A x KBG-A	2	532	97.2
KBG-B x KBG-B	9	1592	94.9
KBG-A x KBG-B	9	1819	81.9
KBG-B x KBG-A	9	1790	90.0
AUB-B x KBG-B	8	927	66.2
KBG-B x AUB-B	4	813	73.9

Table 6. The sex ratio, percent survival to adult stage and F_1 male fertility in A and B strains and cross-matings.

Cross female and male	Number of first instar larvae	Total adults	Percent males	Percent survival to adult stage	Male fertility
AUB-A x AUB-A	1098	669	56.9	60.9	+
AUB-B x AUB-B	1501	1157	53.5	77.1	+
AUB-A x ORL	1230	760	51.1	74.4	+
ORL x AUB-A	1275	806	52.5	71.0	+
AUB-B x ORL	1662	1388	49.8	92.2	-
ORL x AUB-B	701	258	0	36.8	NA
AUB-A x AUB-B	2426	589	0	23.5	NA
AUB-B x AUB-A	3176	1387	48.4	43.7	-
KBG-A x KBG-A	517	371	45.3	71.8	+
KBG-B x KBG-B	1510	758	56.6	50.2	+
KBG-A x KBG-B	1489	692	40.8	39.7	-
KBG-B x KBG-A	1609	639	54.7	46.5	-
AUB-B x KBG-B	614	431	48.3	70.4	+
KBG-B x AUB-B	601	516	52.3	85.9	+

expected, the members of each strain were compatible among themselves. As a second control, each of the AUB strains as crossed to ORL. Both reciprocal crosses between AUB-A and ORL resulted in progeny which were normal in every respect. The AUB-B female X ORL male crosses gave results similar to those obtained from the first series of crosses (Table 4), that is, hatch and development of the F_1 progeny appeared normal, but all F_1 males were sterile. The reciprocal cross, ORL female X AUB-B male, produced results that were different from the initial crosses, in that in contrast to the initial cross where heavy mortality of the F_1 male pupae was observed (sex ratio of 12.2% males) (Table 4), this time all F_1 male pupae died (Table 6).

Crosses between the A and B strains of sympatric origin were conducted. The cross AUB-A female X AUB-B male produced F_1's with the same characteristics as those produced when AUB-B males were mated to ORL. Hatch was high (78.6%), but the % survival to adult was low (23.5%), and all of the F_1 males died in the pupal stage. Results were different for the KBG-A female X KBG-B male cross. Mortality in male pupae was not pronounced; and therefore the sex ratio was closer to normal (40.8% males). Survival to adult stage was also higher (39.7%). In both the reciprocal crosses, AUB-B female X AUB-A male and KBG-B female X KBG-A male produced progeny which were normal in viability, and the sex ratio was normal; but in both cases all F_1 males were sterile.

The final pair of reciprocal crosses between AUB-B and KBG-B established that these two strains were compatible. Progeny resulting from these crosses were comparable in every respect to the control (ORL X ORL). All F_1 males were fertile.

Backcrosses

Three of the four possible backcross combinations were performed, using the AUB strains (Table 7). Hatch was lower in the backcrosses than in the F_1 crosses. In both the F_1 (AUB-A female X AUB-B male) and the F_1 (AUB-B female X AUB-A male) backcrossed to AUB-A the % survival to adult stage was comparable t the A female X B male crosses. Sex ratio was skewed in favor of females, but to a lesser degree than either of the crosses: AUB-B female X ORL male or AUB-B female X AUB-A male. In the cross, F_1 (AUB-A female X AUB-B male) female X AUB-B male, % hatch was also lower than in the F_1 crosses, however % survival to the adult stage was significantly lower. Sex ratio was also skewed in favor of females. Sterility persisted through the backcross, and all of the backcross males were sterile.

Hybridization in Nature

A X B hybrid males can be recognized by microscopic examination of the testes (Figure 8B). Using this technique, it was possible to examine field collected males and determine if they were A X B hybrids. Males were collected

Table 7. The percentage hatch, sex ratio, percent survival to adult stage, and F_1 male fertility in hybrid female backcrosses.

Cross female and male	Number of egg batches	Percent hatch	Total adults	Percent males	Percent survival to adult stage	Male fertility
(AUB-A x AUB-B) F_1 x AUB-A	1	24.7	20	30.0	41.7	-
(AUB-A x AUB-B) F_1 x AUB-B	7	47.3	111	33.3	21.2	-
(AUB-B x AUB-A) F_1 x AUB-A	7	47.4	284	40.5	53.6	-

from the AUB and KBG sites, returned to the laboratory, and the testes were dissected and scored for sterility. Results are presented in Table 8. Of the 143 males from the AUB site and 185 from the KBG site all were normal, indicating that all were either A or B types.

Discussion

A variety of different techniques have been applied to the study of speciation. However, many of the measurable species differences studied contribute little or nothing to reproductive isolation. Hybridization experiments are designed to measure post-mating reproductive isolation directly, and as such have been recognized as the best method available for the study of genic incompatibility (Templeton, 1981).

The hybridization experiments described in this study began with the screening of nine geographic populations in an attempt to detect genetic incompatibility. ORL served as a standard to which all field strains were compared. This strategy eliminated the need of making all possible crosses between field strains. Sterility in F_1 males was observed for some of the crosses between ORL and three of the field strains, ARK, AUB, and KBG while other crosses between these strains and ORL produced normal F_1 progeny. These results established the existence of two sympatric sub-populations at these sites. Crosses between ORL and the remaining six field strains produced normal males in the F_1. It should be noted that these results should not be interpreted as meaning that

Table 8. Survey of AUB and KBG populations for presence of sterile, hybrid males.

Population	Number of males examined	Number of fertile males	Number of sterile males
AUB	143	143	0
KBG	185	185	0

these populations are conspecific with ORL or each other. Under the conditions of these experiments pre-mating isolation between any of these strains would not be detected, nor would post-mating isolation between specific field strains. Crosses between A and B strains developed from the AUB and KBG populations further confirmed the existence of the two types. The incompatibility between the AUB-A X AUB-B and KBG-A X KBG-B crosses proves the existence at these sites, of two reproductively isolated and sympatric populations. Crosses between AUB-B and KBG-B produced normal progeny suggesting that these two populations are conspecific.

Backcrosses of hybrid females to A and B males resulted in a significantly lower hatch than was observed in any of the control matings. The fact that male sterility persists through the backcross indicates that potential gene flow through F_1 females is inhibited.

The examination of field collected males from both sites provided no evidence of hybridization, indicating that some form of pre-mating reproductive isolation separates the A and B populations in nature.

Kitzmiller et al. (1967) used the results of hybridization experiments to assess the degree of relatedness between different species in the A. maculipennis complex. Their results agreed well with phylogenetic estimates based on chromosomal differences. Relatedness is determined by the degree of genetic compatibility with

results ranging from failure of sperm to fertilize eggs to the production of adults in a normal 1:1 ratio but with varying degrees of sterility. The former case indicated a distant relationship, the latter, a close relationship. Applying these criteria to A. quadrimaculatus species A and B, it can be seen that they represent two closely related sibling species. In the cross, species A female X species B male, adults were produced, but the sex ratio was usually distorted in favor of females. Survival through the larval stages was high, but heavy mortality of male pupae was generally observed. In the reciprocal cross adults were produced in a 1:1 sex ratio and survival through all stages was high. All male progeny from both crosses were sterile. Female progeny can be described as semi-sterile since when backcrossed to species A or B, hybrid females produced smaller egg batches and hatch was low. Sterility in male progeny persisted in the backcrosses.

Relationships similar to that between A. quadrimaculatus Species A and B have been described between other sibling species in the genus Anopheles. Within the Palearctic species of the A. maculipennis complex, several species show relationships comparable to the one described here. The cross, A. labranchiae X A. atroparvus, produced sterile males and fertile females, but in this case male fertility was recovered in the F_1 backcross to A. atroparvus males. Sterility in both sexes was observed in the F_1 produced from matings between A. maculipennis and A. atroparvus, whereas

only male progeny were sterile in the cross A. subalpinus Hackett and Lewis X A. gambiae. All possible crosses between members of the A. gambiae Giles complex have been made (Davidson, 1964, Davidson and Hunt, 1973). Some crosses produced only males. Without exception all of the males were sterile, and when produced, the females were fertile. Crosses between A. merus Doenitz females to A. gambiae s.s. or A.arabiensis Patton males produced all male progeny. Likewise, matings between A. melas Theobald females and A. gambiae s.s or A. arabiensis males produced only males. On the other hand, the crosses A. melas female X A. gambiae species D males and A. gambiae species D females X A. merus males produced sex ratios strongly in favor of females (25 and 16.7% males respectively). Mahon and Meithke (1982) report the results of crosses between the three sibling species of A. farauti Laveran. The relationship between these three species parallels that between A. quadrimaculatus A and B. All crosses between A. farauti species 1, 2 and 3 produced sterile male progeny. Sex ratio distortion in favor of females was observed for the crosses A. farauti species No. 1 female X No. 3 male (5% males) and A. farauti no. 3 female X No. 2 male (9% males), but the reciprocals of each produced normal sex ratios. The general pattern of sterile males and fertile females in the F_1 have been reported for a number of other species, including the crosses A. balabacensis Baisas X A. dirus Peyton and Harrison (Baima and Harrison 1980) and A. sinensis Wiedemann X A.

engarensis Kanda and Oguma (Kanda and Oguma 1978). Unidirectional male sterility has been reported in A. culicifaces Giles, where the cross A. culicifaces species A females X species B males results in sterile male progeny but the reciprocal produces fertile males (Miles 1981). In A. coustani Laveran species A and B a similar unidirectional effect has been observed (Coetzee 1983). In this case when A. coustani species B is the female parent, the cross produces sterile male progeny. However, the reciprocal cross results in the production of non-viable eggs.

In conclusion, hybridization studies involving nine geographic strains of A. quadrimaculatus revealed the existence of two reproductively isolated sympatric populations. No evidence of natural hybridization between the two forms was found. These results support the conclusion that A. quadrimaculatus actually exists as two sibling species, provisionally designated A. quadrimaculatus Species A and A. quadrimaculatus Species B.

CHAPTER III

ENZYME POLYMORPHISM AND GENETIC STRUCTURE OF POPULATIONS OF Anopheles quadrimaculatus SPECIES A AND B

Introduction

The European Anopheles maculipennis complex stands as a classic example of sibling species and is cited in almost every written account of the sibling species phenomenon (Mayr, 1963; 1969; 1982; Dobzhansky, 1970; White,1973; Wright, 1978). The entire complex consists of fourteen Holarctic species. Interestingly, the nine Palearctic species are all morphologically identical, or nearly so (sibling species), whereas the Nearctic fauna was, heretofore, thought to be made up of five, closely related, but morphologically distinct species. Recently, hybridization studies have revealed that A. quadrimaculatus Say, one of the Nearctic species, actually consists of two sibling species (Chapter II). These studies demonstrated that the two species exist sympatrically at three of nine localities sampled.

When gene flow is restricted between two populations, differences in the composition of alleles and in their

frequencies within each population may develop. Such differences can be measured by determining the allelic frequencies at a number of loci within each population and comparing them. One way to accomplish this is to measure allozyme frequencies.

The purpose of this study was to measure the genetic variability of A. quadrimaculatus in the southeastern United States, in an attempt to answer several questions. First, how much genetic differentiation exists between the two species and between local populations of each? Second, does the pattern of genetic differences confirm the existence of two species? Third, do allozyme phenotypes occur which can be used to distinguish reliably the two species? Finally, what inferences can be made concerning the phylogenetic relationships between the two species?

Materials and Methods

Adult A. quadrimaculatus were collected from the same nine sites that were sampled in the previous hybridization experiments: in Florida at Ginnie Springs (GIN), at Lake Panasofkee (PAN), (Gainesville) and Kanapaha Botanical Gardens (KBG), and at Lake Panasofkee (PAN); in Alabama, 41 miles west of Auburn on I-85 (AUB), and at Guntersville (GUN); in Mississippi at Skene (SKE); in Arkansas at Stuttgart (ARK) and Bebee (BEB) and in Louisiana at Lake Charles (LAC, Figure 6, Chapter II). One additional site, not sampled in the hybridization experiments, was included,

Lake Seminole in the Florida panhandle at the Florida-Georgia-Alabama state line (Figure 6, Chapter II). The collecting techniques employed were identical to those described in Chapter II. On return to the laboratory collections were sorted on a cold table and stored at -60°C until prepared for electrophoresis. All mosquitoes included in this study were field collected adults.

Determination of species by hybridization was achieved by crossing field collected males to species A females (ORL strain, see Chapter II). If the resulting male progeny were sterile, or no male progeny were produced the parental male was identified as species B, if normal male progeny were produced the male parent was identified as species A.

Electrophoretic techniques for 27 loci in 20 enzyme systems were described in Chapter I. Of these, the following 20 loci were included in this study: Aconitase (ACON, 1 locus), Adenylate kinase (ADK, 1 locus), Catalase (CAT, 1 locus), Glutamate oxaloacetate transaminase (GOT, 2 loci), alpha-Glycerophosphate dehydrogenase (alpha-GPDH, 1 locus), Hexokinase (HK, 2 loci), Hydroxy acid dehydrogenase (HAD, 1 locus), Isocitrate dehydrogenase (IDH, 2 loci), Lactate dehydrogenase (LDH, 1 locus), Malic dehydrogenase (MDH, 1 locus), Malic enzyme (ME, 1 locus), Mannose phosphate isomerase (MPI, 2 loci), Peptidase (PEP, 1 locus), Phosphoglucose isomerase (PGI, 1 locus), Phosphoglucomutase (PGM, 1 locus), and Sorbitol dehydrogenase (SODH, 1 locus). Techniques used for the visualization of the enzymes,

including buffer systems, staining procedures and locus and allele nomenclature are described in Chapter I. In addition, Chapter I contains a description of the genetic basis of isozymes at nine loci. The banding patterns of the remaining isozymes were consistent with a genetic interpretation and agree with previously described phenotypes in other Anopheline species. Thus banding phenotypes (electromorphs) could be scored as genotypes. To insure identity of alleles between populations, a series of gels were run with samples representing each population run concurrently on the same gel in combination with all other populations.

Analyses of allele frequency data were performed using the BIOSYS-1 computer program of Swofford and Selander (1981).

Results

Initially, allele frequencies were calculated under the assumption that A. quadrimaculatus consisted of a single, randomly mating population at each of the ten sites sampled. This assumption was tested by calculating chi-square tests for goodness of fit to Hardy-Weinberg equilibrium for each polymorphic locus. A locus was considered polymorphic if the frequency of the most common allele did not exceed 0.95 (Ayala, et al., 1974). This definition was used throughout this report, unless otherwise stated. Significant deviation from Hardy-Weinberg equilibrium was observed in two of the tests. Chi-square values for Idh-1 and Idh-2 were highly

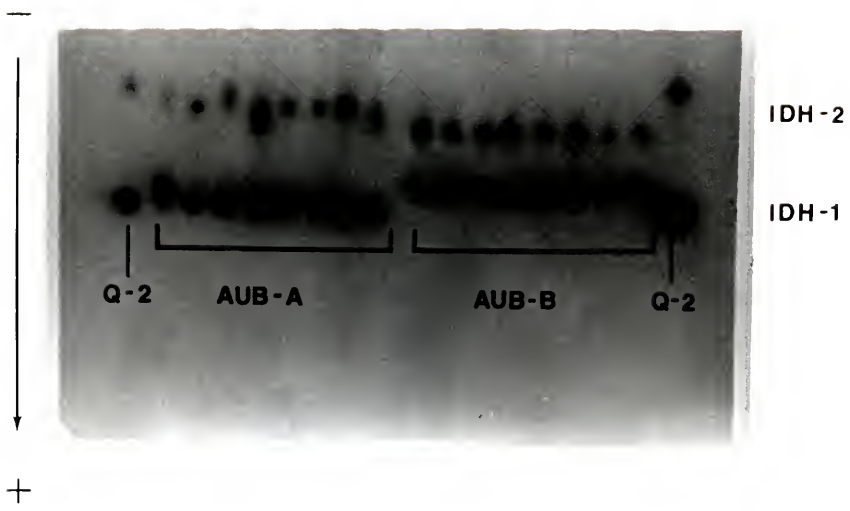
Table 9. Goodness of fit to Hardy-Weinberg expectations for heterozygote frequencies at the Idh-1 and Idh-2 loci in populations of A. quadrimaculatus prior to grouping into species A and B. (D = Selander's (1970) D coefficient for heterozygote deficiency).

	Idh-1[a]				Idh-2[a]				
	N	observed heterozygotes	expected heterozygotes	X^2	D	N	observed heterozygotes	expected heterozygotes	X^2	D
ARK	419	91	140.4	55.3*	-0.352	417	61	116.6	167.2*	-0.477
AUB	425	35	173.6	345.0*	-0.789	410	37	159.5	318.0*	-0.768
KBG	412	83	209.6	462.5*	-0.604	344	119	181.3	81.4*	-0.344
SEM	362	72	163.5	297.8*	-0.560	325	115	169.8	60.0*	-0.323

*P<0.01

[a]Alleles were pooled into two classes, one representing the most common allele, and the other representing all other alleles.

Figure 10. Zymogram of gel stained for IDH (isocitrate dehydrogenase) showing the positions of the diagnostic alleles at the Idh-1 and Idh-2 loci. Q-2 = marker strain serving as control; AUB-A = adult males of species A from the AUB site; AUB-B = adult males of species B from the AUB site.


−
+
IDH-2
IDH-1
Q-2
AUB-A
AUB-B
Q-2

significant at four (ARK, AUB, KBG and SEM) of the ten sites (Table 9). In addition, Selander's (1970) D coefficient is negative in each case, indicating that a deficiency of heterozygotes exists. These data strongly suggest at least two populations at these sites.

The populations at ARK, AUB and KBG were known to be composed of the two sibling species, designated A. quadrimaculatus Species A and B. Differences between the two species in allele frequencies at the IDH loci would account for the deficiency in heterozygotes. To demonstrate this, individual field collected males were positively identified by hybridization, electrophoresed and the gels stained for IDH. A total of 84 individuals from the AUB site and 22 from the KBG site were tested. The Species B males were fixed for a single allele at both the Idh-1 and Idh-2 loci, but species A was polymorphic at these loci. Using the genotypes at these two loci it was possible to correctly identify 32 individuals as species A and 74 as species B.

Figure 10 illustrates a typical IDH zymogram comparing the two species. The diagnostic value of the IDH loci was calculated after Ayala and Powell (1972). Using the genotype at the Idh-1 locus, individuals could be correctly identified as being species A or species B with a probability of 98.61%. The Idh-2 locus provided correct identification at a probability of 98.43%. Neither satisfies the definition of a diagnostic locus, which has been defined by Ayala and Powell (1972) as a locus which provides correct identification at a

probability of 99% or higher. When the two IDH loci are used together, the probability of correct identification is increased to 99.98%. Thus, a tool was provided for rapid identification of individuals which could be grouped into discrete populations whose genetic constitution could then be defined and compared.

Chi-square tests for goodness of fit to Hardy-Weinberg were repeated following grouping assuming that both species represented single panmictic populations at each site. The results for the species A populations are presented in Table 10; species B was, as mentioned, fixed for a single allele at each locus. In two populations (SEM-A and AUB-A) the tests indicate a significant departure from Hardy-Weinberg expectations. Prior to grouping, the IDH genotypes departed significantly from Hardy-Weinberg equilibrium, but after grouping a close-fit to predicted genotypic frequencies was apparent, except as noted for SEM-A and AUB-A.

Table 11 presents allele frequency data for the twenty loci analyzed. Also included are the number of individuals examined per locus for each population. Data for the population of species B at the ARK site is not presented because it was not possible to obtain an adequate sample size representing this population.

Comparing populations of species A with those of species B, the loci having the greatest differences in allele frequencies were Idh-1 and Idh-2. At the Idh-1 locus the 100

Table 10. Goodness of fit to Hardy-Weinberg expectations for heterozygote frequencies at the Idh-1 and Idh-2 loci in populations of A. quadrimaculatus species A. (D = Selander's (1970) D coefficient for heterozygote deficiencies)

	Idh-1[a]					Idh-2[a]				
	N	observed heterozygotes	expected heterozygotes	X^2	D	N	observed heterozygotes	expected heterozygotes	X^2	D
ARK-A	388	92	98.1	1.96	-0.062	386	61	65.4	1.27	-0.068
AUB-A	143	35	42.7	3.88	-0.181	128	37	56.9	17.30*	-0.350
KBG-A	276	83	88.2	1.03	-0.059	208	119	118.7	0.23	0.003
SEM-A	299	72	89.1	7.84*	-0.192	262	115	115.4	0.04	-0.003

*P<0.01

[a] Alleles were pooled into two classes, one representing the most common allele, and the other representing all other alleles.

Table 11. Comparison of gene frequencies among 13 populations of Anopheles quadrimaculatus species A and B. (N = number of individuals studied).

		A. quadrimaculatus sp. A										A. quadrimaculatus sp. B		
Locus	Allele	ARK-A	AUB-A	BEB	GIN	GUN	KBG-A	LAC	PAN	SEM-A	SKE	AUB-B	KBG-B	SEM-B
Acon	N =	81	60	55	62	75	63	151	140	96	91	47	15	62
	$Acon^{84}$	0.006	0.000	0.000	0.032	0.000	0.008	0.003	0.000	0.016	0.005	0.021	0.033	0.024
	$Acon^{94}$	0.012	0.033	0.027	0.048	0.033	0.024	0.026	0.089	0.036	0.016	0.032	0.100	0.040
	$Acon^{100}$	0.932	0.917	0.945	0.831	0.933	0.937	0.934	0.893	0.901	0.945	0.926	0.867	0.895
	$Acon^{106}$	0.025	0.050	0.027	0.056	0.033	0.032	0.033	0.018	0.042	0.033	0.021	0.000	0.040
	$Acon^{113}$	0.025	0.000	0.000	0.032	0.000	0.000	0.003	0.000	0.005	0.000	0.000	0.000	0.000
Adk	N =	130	93	91	106	130	58	88	110	167	91	151	106	58
	Adk^{83}	0.000	0.000	0.000	0.000	0.019	0.009	0.000	0.005	0.003	0.000	0.000	0.000	0.009
	Adk^{100}	0.969	0.973	0.956	0.962	0.938	0.966	0.960	0.982	0.976	0.962	1.000	1.000	0.991
	Adk^{117}	0.031	0.027	0.044	0.038	0.042	0.026	0.040	0.014	0.021	0.038	0.000	0.000	0.000
Cat	N =	115	88	101	163	120	68	159	218	170	126	153	102	51
	Cat^{79}	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.009	0.000	0.000	0.000	0.000
	Cat^{100}	1.000	1.000	1.000	0.997	0.992	1.000	1.000	1.000	0.991	1.000	1.000	1.000	1.000
	Cat^{121}	0.000	0.000	0.000	0.003	0.008	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Got-1	N =	248	118	194	119	167	172	96	245	177	174	146	55	46
	$Got\text{-}1^{84}$	0.010	0.047	0.031	0.025	0.024	0.017	0.021	0.004	0.054	0.017	0.034	0.109	0.043
	$Got\text{-}1^{100}$	0.972	0.945	0.964	0.950	0.973	0.956	0.943	0.982	0.915	0.963	0.829	0.882	0.880
	$Got\text{-}1^{112}$	0.016	0.008	0.005	0.025	0.003	0.015	0.036	0.014	0.025	0.017	0.137	0.009	0.076
	$Got\text{-}1^{120}$	0.002	0.000	0.000	0.000	0.000	0.012	0.000	0.000	0.006	0.000	0.000	0.000	0.000
Got-2	N =	109	63	60	77	77	55	57	89	96	55	43	58	44
	$Got\text{-}2^{38}$	0.417	0.294	0.292	0.370	0.273	0.345	0.447	0.292	0.401	0.418	0.023	0.043	0.080
	$Got\text{-}2^{100}$	0.583	0.698	0.683	0.617	0.714	0.655	0.553	0.685	0.583	0.573	0.965	0.957	0.920
	$Got\text{-}2^{213}$	0.000	0.008	0.025	0.013	0.013	0.000	0.000	0.022	0.016	0.009	0.012	0.000	0.000

Table 11 continued.

Locus	Allele	A. quadrimaculatus sp. A										A. quadrimaculatus sp. B		
		ARK-A	AUB-A	BEB	GIN	GUN	KBG-A	LAC	PAN	SEM-A	SKE	AUB-B	KBG-B	SEM-B
a-Gpdh	N =	128	111	135	58	100	149	94	196	90	117	107	81	37
	a-Gpdh^{74}	0.004	0.000	0.000	0.000	0.005	0.000	0.000	0.000	0.006	0.000	0.009	0.000	0.000
	a-Gpdh^{100}	0.996	1.000	0.996	1.000	0.995	0.997	1.000	0.992	0.994	1.000	0.981	0.988	1.000
	a-Gpdh^{122}	0.000	0.000	0.004	0.000	0.000	0.003	0.000	0.008	0.000	0.000	0.009	0.012	0.000
Had	N =	152	120	94	81	111	123	110	144	112	112	123	107	51
	Had^{56}	0.010	0.008	0.005	0.019	0.005	0.020	0.005	0.021	0.000	0.027	0.012	0.014	0.020
	Had^{100}	0.921	0.896	0.894	0.938	0.887	0.919	0.841	0.885	0.879	0.692	0.943	0.977	0.951
	Had^{156}	0.069	0.096	0.101	0.043	0.108	0.061	0.155	0.094	0.121	0.277	0.045	0.009	0.010
	Had^{189}	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.004	0.000	0.000	0.020
Hk-1	N =	162	110	96	135	118	134	187	197	226	126	177	117	63
	HK-1^{100}	1.000	0.991	1.000	0.996	0.992	1.000	0.992	0.995	0.989	1.000	0.992	0.996	1.000
	HK-1^{122}	0.000	0.005	0.000	0.000	0.000	0.000	0.005	0.003	0.004	0.000	0.003	0.004	0.000
	HK-1^{130}	0.000	0.005	0.000	0.000	0.000	0.000	0.003	0.003	0.007	0.000	0.006	0.000	0.000
Hk-2	N =	162	110	96	135	118	136	187	197	226	126	177	177	63
	HK-2^{100}	1.000	0.991	1.000	0.996	0.996	0.993	0.995	0.997	0.991	1.000	0.992	0.996	1.000
	HK-2^{115}	0.000	0.005	0.000	0.004	0.004	0.007	0.000	0.000	0.002	0.000	0.002	0.004	0.000
	HK-2^{145}	0.000	0.004	0.000	0.000	0.000	0.000	0.005	0.003	0.007	0.000	0.006	0.000	0.000
Idh-1	N =	388	143	186	138	166	276	151	298	299	205	282	136	63
	Idh-1^{73}	0.000	0.000	0.000	0.007	0.000	0.005	0.007	0.002	0.002	0.000	0.000	0.000	0.000
	Idh-1^{85}	0.147	0.161	0.161	0.210	0.130	0.174	0.202	0.174	0.135	0.180	1.000	1.000	1.000
	Idh-1^{100}	0.852	0.822	0.812	0.775	0.867	0.806	0.735	0.800	0.826	0.805	0.000	0.000	0.000
	Idh-1^{110}	0.001	0.017	0.027	0.007	0.003	0.013	0.056	0.023	0.037	0.015	0.000	0.000	0.000
	Idh-1^{129}	0.000	0.000	0.000	0.000	0.000	0.002	0.000	0.000	0.000	0.000	0.000	0.000	0.000

Table 11 continued.

		A. quadrimaculatus sp. A										A. quadrimaculatus sp. B		
Locus	Allele	ARK-A	AUB-A	BEB	GIN	GUN	KBG-A	LAC	PAN	SEM-A	SKE	AUB-B	KBG-B	SEM-B
Idh-2	N =	386	128	204	120	129	208	181	235	262	198	282	136	63
	$Idh\text{-}2^{100}$	0.034	0.078	0.022	0.050	0.016	0.075	0.058	0.068	0.031	0.028	0.000	0.000	0.000
	$Idh\text{-}2^{136}$	0.909	0.715	0.904	0.696	0.938	0.558	0.685	0.562	0.706	0.914	0.000	0.000	0.000
	$Idh\text{-}2^{173}$	0.049	0.195	0.066	0.233	0.043	0.334	0.227	0.323	0.244	0.056	1.000	1.000	1.000
	$Idh\text{-}2^{209}$	0.008	0.012	0.007	0.021	0.000	0.034	0.030	0.047	0.019	0.003	0.000	0.000	0.000
	$Idh\text{-}2^{85}$	0.000	0.000	0.000	0.000	0.004	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Ldh	N =	87	79	101	135	139	41	99	83	101	90	85	124	54
	Ldh^{95}	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.004	0.000
	Ldh^{100}	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.988	1.000	1.000	1.000	0.996	1.000
	Ldh^{107}	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.012	0.000	0.000	0.000	0.000	0.000
Mdh	N =	134	118	135	113	96	140	69	163	114	116	107	119	60
	Mdh^{75}	0.004	0.013	0.004	0.000	0.010	0.004	0.000	0.000	0.013	0.004	0.000	0.004	0.000
	Mdh^{100}	0.948	0.919	0.952	0.978	0.927	0.939	0.942	0.982	0.939	0.940	1.000	0.971	0.983
	Mdh^{125}	0.045	0.064	0.037	0.018	0.057	0.057	0.058	0.015	0.039	0.052	0.000	0.008	0.017
	Mdh^{150}	0.004	0.004	0.007	0.004	0.005	0.000	0.000	0.003	0.009	0.004	0.000	0.017	0.000
Me	N =	135	119	133	99	117	65	66	217	107	135	78	78	59
	Me^{88}	0.000	0.000	0.000	0.010	0.000	0.000	0.000	0.005	0.000	0.004	0.026	0.000	0.000
	Me^{94}	0.048	0.071	0.083	0.131	0.056	0.092	0.076	0.090	0.107	0.063	0.026	0.000	0.008
	Me^{100}	0.948	0.903	0.917	0.859	0.889	0.892	0.924	0.906	0.855	0.933	0.949	1.000	0.922
	Me^{113}	0.004	0.025	0.000	0.000	0.021	0.000	0.000	0.000	0.023	0.000	0.000	0.000	0.000
	Me^{97}	0.000	0.000	0.000	0.000	0.034	0.015	0.000	0.000	0.014	0.000	0.000	0.000	0.000

Table 11 continued.

		A. quadrimaculatus sp. A										*A. quadrimaculatus* sp. B		
Locus	Allele	ARK-A	AUB-A	BEB	GIN	GUN	KBG-A	LAC	PAN	SEM-A	SKE	AUB-B	KBG-B	SEM-B
Mpi-1	N =	104	40	59	66	50	10	95	47	123	62	96	67	40
	$Mpi\text{-}1^{62}$	0.000	0.050	0.000	0.000	0.000	0.000	0.000	0.000	0.008	0.000	0.000	0.000	0.000
	$Mpi\text{-}1^{77}$	0.000	0.000	0.000	0.000	0.030	0.000	0.000	0.000	0.016	0.024	0.000	0.022	0.012
	$Mpi\text{-}1^{79}$	0.048	0.063	0.017	0.038	0.030	0.000	0.079	0.043	0.053	0.065	0.000	0.067	0.050
	$Mpi\text{-}1^{87}$	0.288	0.112	0.347	0.364	0.290	0.500	0.463	0.500	0.500	0.355	0.052	0.187	0.237
	$Mpi\text{-}1^{92}$	0.524	0.462	0.356	0.462	0.490	0.400	0.447	0.362	0.378	0.435	0.427	0.366	0.425
	$Mpi\text{-}1^{98}$	0.100	0.000	0.000	0.076	0.030	0.000	0.000	0.085	0.008	0.048	0.036	0.000	0.000
	$Mpi\text{-}1^{100}$	0.130	0.275	0.271	0.061	0.120	0.100	0.011	0.011	0.037	0.073	0.438	0.284	0.250
	$Mpi\text{-}1^{104}$	0.000	0.037	0.008	0.000	0.010	0.000	0.000	0.000	0.000	0.000	0.047	0.075	0.025
Mpi-2	N =	65	13	85	107	81	39	110	154	117	58	113	99	40
	$Mpi\text{-}2^{83}$	0.000	0.000	0.012	0.005	0.000	0.000	0.000	0.003	0.000	0.000	0.000	0.000	0.012
	$Mpi\text{-}2^{100}$	1.000	1.000	0.982	0.995	1.000	0.962	0.991	0.990	0.991	0.983	1.000	1.000	0.987
	$Mpi\text{-}2^{125}$	0.000	0.000	0.006	0.000	0.000	0.038	0.009	0.006	0.009	0.017	0.000	0.000	0.000
Pep	N =	132	117	99	93	151	127	97	112	108	114	132	109	51
	Pep^{50}	0.008	0.124	0.040	0.032	0.060	0.055	0.021	0.080	0.120	0.035	0.076	0.037	0.020
	Pep^{100}	0.936	0.825	0.924	0.957	0.914	0.917	0.964	0.920	0.838	0.961	0.898	0.927	0.931
	Pep^{163}	0.057	0.051	0.035	0.011	0.023	0.024	0.015	0.000	0.032	0.004	0.015	0.037	0.049
	Pep^{181}	0.000	0.000	0.000	0.000	0.003	0.004	0.000	0.000	0.009	0.000	0.008	0.000	0.000
	Pep^{190}	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.004	0.000	0.000
Pgi	N =	101	114	130	124	102	123	127	170	143	145	135	119	60
	Pgi^{80}	0.000	0.000	0.015	0.000	0.000	0.000	0.004	0.006	0.007	0.010	0.000	0.000	0.000
	Pgi^{94}	0.010	0.004	0.031	0.004	0.000	0.004	0.008	0.018	0.003	0.014	0.015	0.008	0.000
	Pgi^{100}	0.990	0.996	0.931	0.996	1.000	0.996	0.988	0.976	0.983	0.976	0.985	0.992	1.000
	Pgi^{105}	0.000	0.000	0.023	0.000	0.000	0.000	0.000	0.000	0.007	0.000	0.000	0.000	0.000

Table 11 continued.

		A. quadrimaculatus sp. A										*A. quadrimaculatus* sp. B		
Locus	Allele	ARK-A	AUB-A	BEB	GIN	GUN	KBG-A	LAC	PAN	SEM-A	SKE	AUB-B	KBG-B	SEM-B
Pgm	N =	115	103	110	129	93	94	127	186	141	66	136	96	56
	Pgm^{86}	0.061	0.087	0.032	0.047	0.048	0.043	0.035	0.019	0.060	0.023	0.018	0.010	0.027
	Pgm^{93}	0.000	0.000	0.000	0.000	0.005	0.000	0.004	0.000	0.000	0.000	0.000	0.000	0.000
	Pgm^{100}	0.887	0.835	0.914	0.853	0.871	0.878	0.866	0.922	0.862	0.947	0.813	0.719	0.679
	Pgm^{114}	0.048	0.073	0.050	0.093	0.070	0.064	0.083	0.059	0.071	0.030	0.165	0.250	0.241
	Pgm^{118}	0.000	0.000	0.000	0.008	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
	Pgm^{121}	0.000	0.005	0.005	0.000	0.005	0.011	0.012	0.000	0.004	0.000	0.004	0.010	0.000
	Pgm^{129}	0.004	0.000	0.000	0.000	0.000	0.005	0.000	0.000	0.004	0.000	0.000	0.010	0.000
Sodh	N =	108	80	100	128	117	77	110	155	145	117	123	91	39
	$Sodh^{63}$	0.009	0.106	0.005	0.000	0.000	0.000	0.005	0.010	0.000	0.030	0.008	0.000	0.013
	$Sodh^{100}$	0.977	0.862	0.970	0.992	0.991	1.000	0.973	0.987	0.990	0.953	0.939	0.989	0.987
	$Sodh^{113}$	0.014	0.031	0.020	0.008	0.009	0.000	0.018	0.003	0.010	0.017	0.053	0.011	0.000
	$Sodh^{138}$	0.000	0.000	0.005	0.000	0.000	0.000	0.005	0.000	0.000	0.000	0.000	0.000	0.000

allele predominated in populations of species A, whereas populations of species B were fixed for the 85 allele. A similar pattern was presented for the Idh-2 locus. The 136 allele was most frequent in species A populations while populations of species B were fixed for the 173 allele.

There were four loci at which both species shared the most common allele, but differed at alleles with intermediate frequencies. At the Got-2 locus both species shared the 38 allele, but the average frequency of this allele in species A was .335 while its frequency in species B was .049. There were eight alleles at the Mpi-1 locus with both species sharing the frequent allele. In populations of species A the 87 allele occurred with a frequency of .372, whereas, in species B its frequency was .159. The 100 allele was the second most common allele in species B with a frequency of .324, the frequency of this allele in species A was .109. With the exception of ARK-A and AUB-A, the 114 allele at the Pgm locus was the second most common allele in populations of both species, but occurred at very different frequencies. In species B the 114 allele occurred at a frequency of .219, but has a frequency of only .064 in species A. Likewise at the Me locus the 100 allele was the most common allele in both species, with the 94 allele being the next most frequent, (absent in KBG-B) with an average frequency of .082 in species A, but only .011 in species B.

Polymorphisms existed at six loci which showed little differentiation between the two species. These included

Acon, Got-1, Had, Mdh, Mpi-2 and Pep. Eight loci: Adk, Cat, Gpdh, Hk-1, Hk-2, Ldh, Sodh, and Pgi were not polymorphic by the 0.95 criterion.

Measures of genetic variability, including mean numbers of alleles per locus, percent polymorphic loci and mean heterozygosity are presented for each population in Table 12. These results indicate that populations of species B are less variable genetically than those of species A. The number of electrophoretically detectable alleles, occurring at a frequency of at least 1%, varied from one (Cat, Hk-1, Hk-2, Ldh) to eight (Mpi-1). The mean number of alleles per locus over all populations averaged 2.9 for species A and 2.3 for species B. Species A was polymorphic at 50.5% of the loci studied and species B at only 31.7%. Species B had a lower mean heterozygosity as well, with 10.3% of its genes, on average, in the heterozygous condition, while species A had a heterozygosity of 15.9%.

Estimates of genetic distance and similarity between species and between populations within species, were made using the I and D statistics as defined by Nei (1978). I and D values for all pairwise comparisons are presented in Table 13. The average distance (D) between local populations of species A was .005 ($\pm$ .003) and between populations of species B was .002 ($\pm$ .014). Genetic identity and distance coefficients demonstrate a high degree of differentiation between the two species relative to that of local populations within species. Genetic distance is much higher between

Table 12. Genetic variability in populations of Anopheles quadrimaculatus species A and B.

Population	Mean sample size per locus	Mean number alleles per locus	Percentage of loci polymorphic[a]	Mean heterozygosity[b]
		SPECIES A		
ARK-A	152.1	2.7		0.127
	(19.9)	(0.3)	50.0	(0.037)
AUB-A	96.3	2.8		0.180
	(7.2)	(0.3)	60.0	(0.042)
BEB	113.2	2.8		0.143
	(9.4)	(0.3)	50.0	(0.038)
GIN	109.4	2.8		0.162
	(6.5)	(0.3)	45.0	(0.044)
GUN	112.8	2.9		0.137
	(6.7)	(0.3)	55.0	(0.037)
KBG-A	107.9	2.8		0.159
	(14.3)	(0.3)	50.0	(0.043)
LAC	118.0	2.8		0.162
	(8.0)	(0.3)	50.0	(0.042)
PAN	167.8	2.9		0.155
	(13.7)	(0.2)	45.0	(0.043)
SEM-A	151.0	3.4		0.175
	(13.4)	(0.3)	55.0	(0.040)
SKE	116.2	2.8		0.148
	(9.5)	(0.3)	45.0	(0.042)
MEAN	124.5	2.9	50.5	0.159
		SPECIES B		
AUB-B	134.6	2.5		0.103
	(13.9)	(0.3)	40.0	(0.038)
KBG-B	96.6	2.3		0.102
	(6.8)	(0.3)	25.0	(0.042)
SEM-B	53.0	2.2		0.105
	(2.0)	(0.3)	30.0	(0.041)
MEAN	94.7	2.3	31.7	0.103

[a]0.95 criterion
[b]Hardy-Weinberg expected

Table 13. Genetic distances and identities among 13 populations of Anopheles quadrimaculatus species A and B. Genetic distances for each pairwise comparison are located below the diagonal; genetic identities above.

Population	1	2	3	4	5	6	7	8	9	10	11	12	13
1 ARKA	*****	0.995	0.895	0.998	0.997	0.999	0.993	0.898	0.996	0.991	0.995	0.900	0.997
2 AUBA	0.005	*****	0.920	0.996	0.995	0.996	0.993	0.919	0.991	0.991	0.992	0.920	0.991
3 AUBB	0.111	0.084	*****	0.904	0.919	0.896	0.925	0.998	0.914	0.923	0.907	0.997	0.893
4 BEB	0.002	0.004	0.101	*****	0.995	0.999	0.994	0.905	0.994	0.992	0.995	0.907	0.996
5 GIN	0.003	0.005	0.085	0.005	*****	0.996	0.999	0.923	0.999	0.998	0.998	0.925	0.994
6 GUN	0.001	0.004	0.110	0.001	0.004	*****	0.993	0.899	0.994	0.991	0.995	0.901	0.997
7 KBGA	0.007	0.007	0.078	0.006	0.001	0.007	*****	0.929	0.999	1.000	0.999	0.932	0.991
8 KBGB	0.108	0.084	0.002	0.100	0.080	0.107	0.073	*****	0.919	0.928	0.913	1.000	0.895
9 LAC	0.004	0.009	0.090	0.006	0.001	0.006	0.001	0.085	*****	0.997	0.999	0.922	0.996
10 PAN	0.009	0.009	0.080	0.008	0.002	0.009	0.000	0.075	0.003	*****	0.998	0.930	0.991
11 SEMA	0.005	0.008	0.097	0.005	0.002	0.005	0.001	0.092	0.001	0.002	*****	0.915	0.994
12 SEMB	0.105	0.083	0.003	0.098	0.078	0.105	0.071	0.000	0.081	0.073	0.089	*****	0.898
13 SKE	0.003	0.009	0.114	0.004	0.006	0.003	0.009	0.111	0.004	0.010	0.006	0.108	*****

species at the same locality than among the same species at different localities.

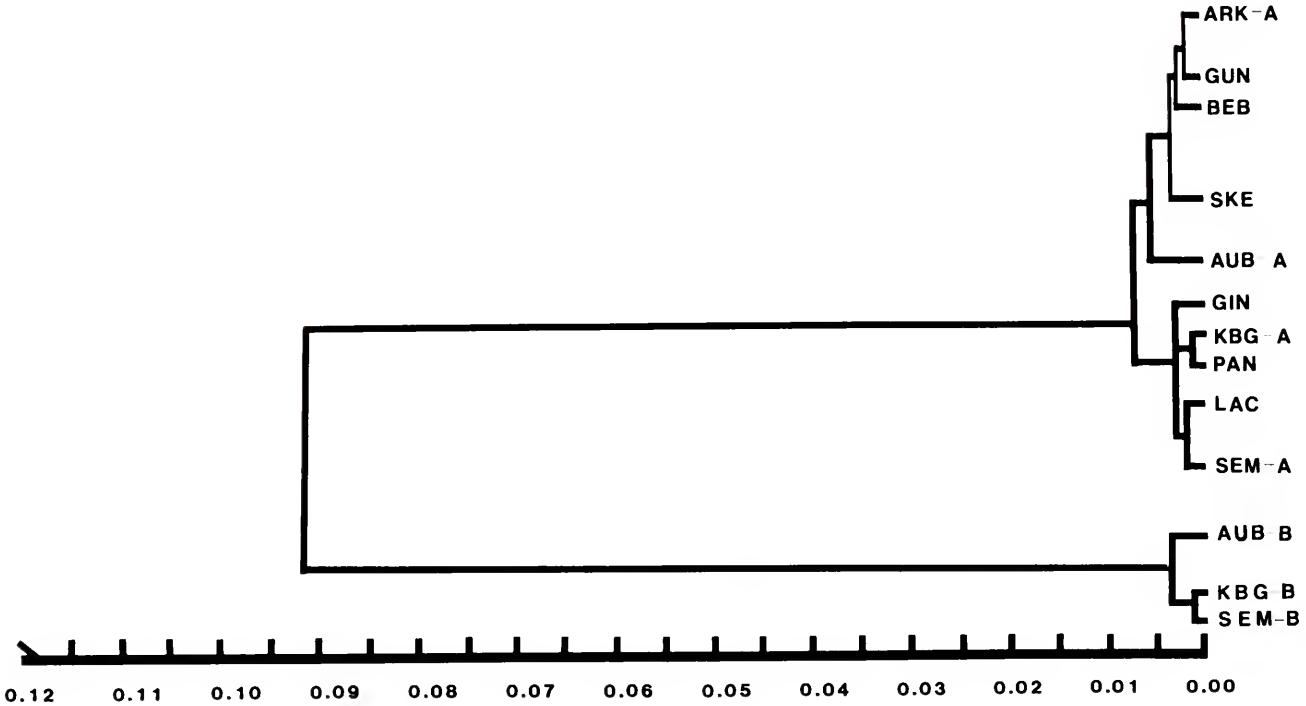
A cluster analysis of the matrix of D values, using the unweighted pair-groups method with arithmetic averages (UPGMA, Sneath and Sokal, 1973), produced the dendogram illustrated in Figure 11. The dendogram had two clusters at a distance level of .092. One cluster contained the three populations of Species B, and the other ten populations of Species A.

Discussion

The data from this study confirm the existence of two sibling species in what was formerly known as the single species, A. quadrimaculatus. Analysis of genotypic frequencies at two IDH loci revealed a highly significant deficiency of heterozygotes at four of the sites sampled. This phenomenon, known as the "Wahlund effect" (Crow and Kimura, 1970), is interpreted as resulting when reproductively isolated populations occur sympatrically and are sampled as a single population. These data alone provide very strong evidence for the existence of two species (Makela and Richardson, 1977; Bullini and Coluzzi, 1982).

The presence of Species A individuals heterozygous at the IDH loci suggested that a limited amount of gene flow might occur between the two proposed species. In fact, it was revealed that species B was fixed for a single allele at both IDH loci and species A was polymorphic and included the

Figure 11. Dendogram constructed from values for D between populations of Anopheles quadrimaculatus species A and B. Dendogram was constructed using the UPGMA method of Sneath and Sokal, 1973.


ARK-A
GUN
BEB
SKE
AUB A
GIN
KBG A
PAN
LAC
SEM-A
AUB B
KBG B
SEM-B
0.12
0.11
0.10
0.09
0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0.00

same two alleles for which species B was fixed, albeit at relatively low frequencies.

A comparison of populations of the two species yielded an average value for the Nei's distance coefficient of 0.092 (± .014). This value is much smaller than the value between sibling species of Drosophila (D = 0.60) reported by Ayala (1975). Average genetic distance between mosquito sibling species are generally lower than those found in Drosophila. The values reported in the literature for various Anopheline sibling species are summarized in Table 14. The average genetic distance between those members of the Palearctic A. maculipennis complex which have been studied (excluding A. melanoon x A. sacharovi) is 0.183. This is substantially larger than the value between A. quadrimaculatus species A and species B. Low values for genetic distance have been reported in the A. gambiae complex (eg. A. gambiae x A. arabiensis, D = 0.070). Extremely low values for D have been reported between some members of the A. marshallii complex (eg. A. marshallii species A x species B, D = 0.045 and A. marshallii species A x species C, D = 0.029). These values are comparable to the genetic distance between local populations of A. maculipennis, D = 0.032 (Bullini and Coluzzi, 1982). Bullini and Coluzzi (1973) observed that low values for genetic distance, as in the A. gambiae complex, are associated with higher levels of chromosomal divergence. They suggest that the speciation process in such groups is

Table 14. Genetic distance (D) between sibling species of mosquitoes in the genus Anopheles.

SPECIES COMPARISON	$\bar{D}$	REFERENCE
Anopheles maculipennis complex		Bullini and Coluzzi, 1982
A. messae x A. subalpinus	0.119	
A. subalpinus x A. melanoon	0.154	
A. subalpinus x A. maculipennis	0.162	
A. melanoon x A. maculipennis	0.228	
A. labranchiae x A. atroparvus	0.250	
A. melanoon x A. sacharovi	0.526	
Anopheles gambiae complex		Bullini and Coluzzi, 1982
A. gambiae x A. arabiensis	0.070	
Anopheles marshallii complex		Lambert, 1983
A. marshallii sp. A x sp. B	0.045	
A. marshallii sp. A x sp. C	0.029	
A. marshallii sp. A x sp. E	0.118	
A. marshallii sp. B x sp. C	0.107	
A. marshallii sp. B x sp. E	0.220	
A. marshallii sp. C x sp. E	0.128	
Anopheles quadrimaculatus complex		
A. quadrimaculatus sp. A x sp. B	0.092	

different than in groups with higher genetic distances, and lower levels of chromosomal divergence, as in members of the European A. maculipennis complex (Bullini and Coluzzi, 19823). Templeton (1981), however points out that the difficulty in such interpretations is the fact that it is impossible to distinguish whether these differences are responsible for the speciation event or are consequences of evolution subsequent to speciation. Genetic distance values may only indicate how recently a speciation event has occurred, being smaller between species which have more recently diverged (Avise, Smith and Ayala, 1975; Carson, 1976).

Conclusions can be drawn regarding the speciation process in this case. There are several facts revealed in the data in this dissertation which suggest that A. quadrimaculatus species A is the ancestral species and that species B evolved from it via a founder event. The genetic distance between the two species is small, relative to the distance values reported between mosquito sibling species, which generally range from 0.10 to 0.30 (Bullini and Coluzzi, 1982). Low values for genetic distance have been observed between even morphologically distinct species recently separated by founder events (Sene and Carson, 1977). These results conflict with conventional thinking which would predict large genetic distances resulting from a "genetic revolution" (Mayr, 1954) produced by the founder event. Templeton (1980) suggests that founder events are more likely

to affect only a small number of genes, while the majority of the genome is unaffected (Templeton, 1980a). In fact, there is evidence that enzyme coding loci are relatively insensitive markers of speciation (Templeton, 1980b).

Populations which go through a small bottleneck experience a decline in genetic variability. The magnitude of the reduction was thought to be substantial, with only a small proportion of the original genetic variability left (Mayr, 1963). Nei, et al. (1975) studied the problem quantitatively and determined the loss of variability to be much smaller when population size increases follow the bottleneck. Table 12 summarizes the data on genetic variability in species A and B. Species B is less variable genetically than species A. Species A has a mean heterozygosity of 15.9% whereas species B has a heterozygosity of 10.3%. The difference is modest, but fits the level of decline predicted by Nei, et al. (1975). The reduction in heterozygosity associated with founder populations is generally attributed to the loss of low frequency alleles by drift. The allelic composition of populations of species B lack many of the low frequency alleles found in populations of species A. Whereas, with the exception of one rare allele (Pep^{190}, $p = 0.004$), populations of species B contain no alleles not also present in populations of species A.

In conclusion, the electrophoretic data confirm the existence of a sibling species. The genetic composition of this new species suggest that it evolved from the ancestral population through a small bottleneck, the founding population may have consisted of less than ten individuals.

CONCLUSIONS

In summary, the results of this study prove the existence of a new sibling species of Anopheles quadrimaculatus (Say). This report represents the first description of a sibling species in the Nearctic Anopheline fauna. This discovery is consistent with findings from a large number of workers that Anopheline species frequently evolve without developing significant morphological differences.

The proof given here, for the existence of the new species, is two-fold. Hybridization experiments revealed that three of the nine populations surveyed existed as two, reproductively isolated, sympatric populations. Reproductive isolation was determined by mating studies which identified male hybrid sterility. Attempts at identifying naturally occurring hybrids at two of the sites failed, indicating that a pre-mating mechanism,probably behavioral, maintains reproductive isolation between these two species. A survey of allozymic variation at twenty gene loci produced data which supported the existence of a sibling species complex. At two loci, a significant deficiency of heterozygotes was revealed in the same three populations identified as being mixed in the hybridization experiments. The genotypes at

these two loci could be used in distinguishing individuals of the two species. The two species were tentatively designated A. quadrimaculatus Species A and B.

The patterns of the genetic makeup of each species were compared and a hypothesis concerning the phylogenetic relationship between them was made. The evidence indicated that species A is the ancestral species and that species B evolved from it through a founder event.

There are five species, in addition to A. quadrimaculatus, which belong to the Nearctic branch of the Anopheles maculipennis complex. The results of this study indicate that each of the remaining four should be more closely studied to determine if additional sibling species exist in this interesting species group.

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Biographical Sketch

Gregory Charles Lanzaro was born on October 2, 1950 in New York City, New York. He graduated from Kansas State University in 1972, with the degree of Bachelor of Science. After graduation he served as a high school teacher of biology at Omaha, Nebraska and New Haven, Connecticut. In 1975 he enrolled in graduate school at the University of Arizona, where he obtained a Master of Science degree in Entomology in 1978. In 1980 he began work for the Doctor of Philosophy degree at the University of Florida. He is an active member in four national scientific societies. At present he serves as Assistant Medical Entomologist in the Department of Entomology of Mississippi State University.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Jack A. Seawright, Chairman
Associate Professor of
Entomology and Nematology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Sudhir K. Narang
Adjunct Associate Professor of
Entomology and Nematology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Donald W. Hall
Professor of Entomology and
Nematology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Stanley C. Schank
Professor of Agronomy

This dissertation was submitted to the Graduate Faculty of the College of Agriculture and to the Graduate School, and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

December 1986

Jack L. Fry

Dean, College of Agriculture

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